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Navarre Beach, Florida, 18-20 October 1983

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The ORD internal report is a summary of proceedings of the Workshop on Biodegradation Kinetics organized to examine future directions and focus of scientific investigations of the kinetic aspects of microbial degradation rates of organic chemicals in natural environments. Position papers cover the following topics: (1) statistical and experimental requirements for modeling decay curves; (2) the "second order" aproach assumption, limitations and research needs; (3) factors controlling biodegradation rates in microbial communities; (4) application of uptake and mineralization kinetics; (5) relationships between chemical structure and biodegradation rates; and (6) extrapolation of laboratory biodegradation data to the field. Discussions within each session are summarized by panel members in reports that include a concensus of the direction and extent of research required for the description of biodegradation rates of xenobiotic chemicals in natural environments.

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NAVARRE BEACH, FLORIDA
18-20 OCTOBER 1983

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FOREWORD

The proliferation of organic chemicals, as a result of the need for new and better agricultural and industrial chemicals, increases the potential for environmental pollution, particularly in aquatic habitats. Inherent in any fate study is the need for an accurate assessment of the potential for the microbial degradation of the pollutant. However, there is little information on the rates at which biodegradation will occur in the environment. Furthermore, kinetic data from laboratory and field studies are often based on assumptions that have no consensus or sound scientific basis. Thus, the microbiologist currently has no consistent guidelines regarding appropriate techniques for the determination of biodegradation rates in natural environments, nor have microbiologists, in general, provided the chemical industry and regulatory agencies with evaluations of the effect of environmental parameters and chemical structures on biodegradation of processes mediated by microbial populations.

This Workshop on Biodegradation Kinetics is a follow-up to a workshop held in Gulf Breeze, Florida, and sponsored by the Environmental Research Laboratory at Gulf Breeze. As was true for the first workshop, this workshop on biodegradation kinetics was organized to offer representatives from government, academia, and industry a forum for the examination of key issues regarding the future direction and focus of scientific investigations in this field. Participants addressed a number of questions of primary concern: What are the differences and similarities between biodegradation rates in aquatic, terrestrial, and laboratory environments? What methodological criteria must be established to provide interchangeable degradation information? What is the potential for a particular environment or its laboratory simulation to dispose of a polluting chemical, and at what rate will it occur? We hope that this publication of the workshop proceedings will provide an up-to-date reference for professionals concerned with the fate, regulation, and production of potential environmental pollutants.

Henry F. Enos

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ABSTRACT

This workshop, held October 18-20, 1983, at Pensacola Beach, Florida, focused on pertinent issues related to the scientific investigation of the microbial degradation rates of organic chemicals in natural environments. Participants discussed methodological criteria for these investigations and the need for concentrating on the kinetic aspects of biodegradation. Position papers dealing with the following topics were presented in open sessions: (1) Statistical and Experimental Requirements for Modeling Decay Curves; (2) The "Second Order" Approach Assumption, Limitations and Research Needs; (3) Factors Controlling Biodegradation Rates in Microbial Communities; (4) Application of Uptake and Mineralization Kinetics; (5) Relationships between Chemical Structure and Biodegradation Rates; and (6) Extrapolation of Laboratory Biodegradation Data to the Field. Discussions within each session are summarized by the panel members in reports that include a consensus of the direction and extent of research required for the description of biodegradation rates of xenobiotic chemicals in natural environments. These proceedings conclude with a summary report and suggestions for future research in biodegradation kinetics. This report is submitted in fulfillment of Contract No. CR 810789 by Gulf Coast Research Laboratory in conjunction with Georgia State University Cooperative Agreement R809370 under the sponsorship of the U.S. Environmental Protection Agency. This report covers a period from April 4, 1984 to May 13, 1984, and work was completed as of June 30, 1984.

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The assistance of Ms. Gail Seidler, Gulf Breeze Environmental Research Laboratory, and the Georgia State University Cooperative Agreement Office at Gulf Breeze, in preparing material before the Workshop is gratefully acknowledged.

I. Workshop Organization

A. Objectives

1. To evaluate the utility and limitations of approaches currently used for the derivation of environmentally meaningful biodegradation rates from laboratory studies.

2. To identify the future research required for the improvement of our approaches in the development of a strategy for the extrapolation of laboratory information on the kinetics of biodegradation to complex environmental situations.

B. Working Concept

Biodegradation studies, in general, provide decay curves for a chemical by using a particular method and inoculum. Decay curves vary under different inocula or different experimental conditions. Application of this laboratory-derived biodegradation information to the field requires that degradation rates be calculated from the decay curves. This is usually accomplished by developing mathematical rate equations or kinetic expressions that model the decay curves. Because of uncertainties about the factors that control the metabolic diversity and degradative potential of natural communities of microorganisms, certain assumptions about the activity, concentration, and substrate affinity of the biological catalysts within these communities and about the effect of environmental parameters (sorption, temperature, multiple substrates, etc.) on their activity must be made if useful kinetic expressions are to be developed and the key, we believe, to measuring biodegradation rates in the laboratory that can be used to predict the biological fate of a chemical in the field. A critical evaluation of the logic associated with the development of each assumption and the research needed to validate the assumptions was the working theme for this workshop.

C. Workshop Structure

The workshop was divided into six sessions:

1. Statistical and Experimental Requirements for Modeling Decay Curves
2. The "Second Order" Approach: Assumptions, Limitations, and Research Needs
3. Factors Controlling Biodegradation Rates in Microbial Communities
4. Application of Uptake and Mineralization Kinetics
5. Relationships Between Chemical Structure and Biodegradation Rates
6. Extrapolation of Laboratory Biodegradation Data to the Field

Each session was initiated with several 10-minute talks that briefly summarized the status, limitations, and research needs of a particular research approach used to study biodegradation kinetics. The talks, presented below, were intended to provide representative data upon which subsequent discussion was based. Position statements and suggestions were encouraged.

The objective of each session was to provide the session chairperson and a panel of 3-5 individuals with interpretations, strategies, theories, recommendations, and priorities that they could use to prepare a report to the workshop coordinator. Session reports were presented to the workshop participants for further discussion and revision in a general plenary session. The final panel reports are presented below.

D. An EPA Perspective by Robert Brink, U.S. EPA

My comments will be directed to the needs of the Office of Toxic Substances (OTS) of EPA, although I am sure that any advances that can be made in the ability to estimate environmentally realistic biodegradation rates will be of value to many others in EPA and elsewhere.

The Toxic Substances Control Act (TSCA), under which OTS operates, applies to all chemical substances not covered by prior acts and allows for the regulation of those chemical substances that may or do present an unreasonable risk of injury to human health or the environment.

An evaluation of the risk or likelihood that a chemical may cause an adverse effect involves a determination of the hazard or adverse effects that may occur if there is exposure and an assessment of the exposure possibilities.

An exposure assessment considers the sources, pathways, concentrations and potentially exposed populations. Chemical fate aspects of the exposure assessment evaluate the transport and transformation possibilities following release of the chemical to the environment and the fate conclusions are important elements of the pathways and concentrations part of the exposure assessment.

An assessment of the fate of a chemical released to the environment will depend, in part, upon laboratory data that can be used to evaluate the properties and processes that influence transport and transformations. In actual practice, fate assessment includes a consideration of existing data from reliable literature citations and estimation techniques that should be considered and that may be used either to avoid unnecessary testing or to guide the proper selection of laboratory testing. The overall approach is summarized in Table 1 which lists, from top to bottom, the properties and processes for which data may be needed and, from left to right, the various levels of data input. In this scheme, laboratory data at one level will be required only when data from a lower level are insufficient to answer relevant exposure assessment questions. The testing may begin at any appropriate spot. For example, if it is already known that the only significant transformation processes will be biodegradation studies in soil and water, the laboratory testing might begin with biodegradation studies that will provide kinetic information and data on potential intermediates of concern. It is also highly unlikely that any given chemical would require testing for all or even a high percentage of the properties or processes. Existing knowledge estimation techniques and the early base-set tests will provide information that will preclude much of the other testing.

Environmentally realistic estimations of chemical concentrations of nonpersistent substances require valid kinetic data with calculations of half-lives under relevant environmental scenarios. For hydrolysis, the data from the base-set will usually be sufficient to calculate environmental half-lives, although there may be circumstances, such as environmental extremes of pH, that will dictate a need for upper tier simulations to obtain better rate data. For selected environments, it may be necessary to conduct kinetic studies at an upper tier, with simulations of particular environmental variables, to obtain better rate values for leaching, evaporation, and photodegradation. It is unlikely that the base-set biodegradation tests will provide sufficient information for valid extrapolation to environmental rates and it is anticipated that any determination of environmentally relevant biodegradation rate data will require upper tier kinetic studies. It should be emphasized that upper tier tests for rate determinations are justified only for significant transfer transformation processes. For the organic compounds evaluated by EPA, transformations in soil and water by micro-organisms are often the most significant transformation processes.

The base set of fate tests will indicate the dominant transformation processes that a chemical may undergo in natural environments. In some cases, there will be indications of whether an organic substance is degraded essentially to inorganic compounds (e.g. carbon dioxide and water) plus normal metabolic intermediates. If a substance is extremely persistent, this will be evident from the lack of reaction by any of the transformation processes. For all of the situations where a chemical substance is observed neither to persist nor to clearly degrade to innocuous inorganics and metabolites, the investigator should be concerned with the question of persistent and potentially toxic transformation products. In some cases, a good background in chemistry and microbiology will enable the investigator to make informed guesses about the potential products, but testing will be required to confirm those guesses. The purposes of the degradation pathways testing are twofold. One question to be answered is whether a toxic substance, when degraded, shows concomitant loss of that toxicity. This is a combined transformation and effects problem. The other question, which involves analytical chemistry, deals with the identification of the products of environmental transformation.

It is very important to us, in OTS, to be able to do better in assessing the biodegradation rates of organic chemicals. This must be accomplished by the use of a series of site-specific microcosms that have been shown to replicate, to some necessary precision, the transformation rates in the simulated environments. I hope that won't be necessary. It would be preferable to be able to use some relatively simple, generic, bench-scale laboratory procedures that would provide biodegradation rate constants that could be translated into rates for specific environments by mathematical manipulations that account for the relevant environmental factors that will influence the rates. We hope that this workshop will consider the possibilities and provide recommendations on the best research to pursue over the next few years to achieve that goal.

TABLF I CHEMICAL FATE TESTING SCHEME

Data Source / Property or Process	Citable Literature Data	Calculations or Estimates	Base-Set Tests	Kinetic Studies (Rates)	Degradation Pathways	Site Simulation with Microcosms
Physical/Chemical Properties						
Absorption, uv/visible spectra			*	X	X	X
Boiling Temperature			*	X	X	X
Density/Relative Density			*	X	X	X
Dissociation Constants in Water			*	X	X	X
Henry's Law Constant			*	X	X	X
Melting Temperature			*	X	X	X
Particle Size Distribution/Fiber Lngth.			*	X	X	X
Partition Coefficient (n-Octanol/Water)			*	X	X	X
pH of Water Soln or Suspension			*	X	X	X
Water Solubility			*	X	X	X
Vapor Pressure			*	X	X	X
Transport Processes						
Evaporation from Water					X	
Evaporation from Soil					X	
Adsorption/Desorption			*		X	
Uptake by Biota					X	
Transformation						
Biodegradation			*			
Photodegdn.-Sunlight Photolysis			*			
-Oxidn by OH radical or O_3			X			
-Indirect			X			
Complex Formation			*			
Hydrolysis			*			

Inappropriate and unnecessary data elements are indicated by X marks within the boxes.

* Methods in the August, 1982 et seq. OTS Guidelines, published by NTIS.

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STATISTICAL AND EXPERIMENTAL REQUIREMENTS FOR MODELING DECAY CURVES

Objective: To discuss the limitations and possibilities for statistically verifying that a kinetic expression appropriately describes a decay curve or set of decay curves.

Biodegradation rates are commonly determined from decay curves assessed by using certain statistical treatments of the data. Improper or insufficient use of statistical methods and incomplete data sets, however, frequently results in equivocal assessments of degradation rates. What statistical analysis is necessary to verify that a kinetic expression appropriately describes a decay curve? What experimental criteria (replication, sampling, data points, precision, time course, etc.) should be established to assure the completeness of data sets used for statistical analysis? When should nonlinear or linear regressions be applied, and what advantages do they offer? At what point does the number of outlying data points mean that a model does not fit the data? What logic and discriminative framework should be used to select models that fit the experimental data?

Panel Members: Joe Robinson (chairperson), Montana State University
David Currie, McGill University
Walter Maier, University of Minnesota

PROBLEMS IN NON-LINEAR PARAMETER ESTIMATION

David Currie

Introduction

In recent years the use of nonlinear models has become increasingly common in biological studies. The statistical treatment of such models presents several difficulties which are not encountered in ordinary linear regression analysis. As a result, uncautious treatment of nonlinear models can often produce very poor parameter estimates and model predictions. It is the purpose of this paper to describe three pitfalls in fitting nonlinear models which are often observed in biological studies. I shall concentrate specifically on the problem of obtaining the best estimates of the model parameters (i.e., constants). A good, readable introduction to the basics of regression analysis and some of its other potential problems is given by Gujarati (1978) and more advanced treatments will be found in Draper and Smith (1981) and Beck and Arnold (1977).

In seeking the "best" estimates of model parameters, I specifically mean estimates which are both accurate (i.e., unbiased) and precise (i.e., having low variance). The precision of estimates almost entirely determines one's ability to test hypothesis. Imprecision undermines the ability to reject the null hypothesis and increase the probability of retaining the null hypothesis when it is in fact false (Type II error). More insidious yet is the effect of inaccuracy (i.e., bias) estimation. Bias is the systematic tendency to overestimate or underestimate a population mean value, and can cause differences among hypothesis to appear to be statistically significant, when they are in fact entirely spurious. In the discussion which follows, I shall consider three common practices which tend to yield inaccurate and imprecise parameter estimates: poor experimental design, inappropriate fitting techniques, and inappropriate model choice.

POOR EXPERIMENTAL DESIGN

The design matrix, that collection of values of the independent variable (x) at which the experimenter decides to take observations, is rarely given much explicit consideration before an experiment is carried out. It can be shown (Draper and Smith 1981) that, for any model, the variance of parameter estimates will depend upon two things: the magnitude of the experimental error (which the experimenter often cannot control) and the design matrix (which usually can be controlled).

The effect of the design matrix is most easily illustrated with a linear relationship (Figure 1). If one wishes to estimate the slope of a straight line (the heavy line in the center of the figure) with a given amount of experimental error around the line (depicted by the vertical bars), then taking observations over a relatively narrow range of x values (interval A) will lead to a much broader confidence interval around the estimate of the slope than will a wider spread of observations (interval B).

The situation is consideraly less obvious when a model is nonlinear. This is due to the fact that estimates of nonlinear parameters depend upon the placement of observations with respect to specific points on the curve. Consider, for example, a simple exponential decay model:

$$y = \alpha e^{-bx}$$

where y is usually the measured amount of a decaying substance, and x is an independent variable such as time or distance. Suppose that α, the initial quantity of substance y, is known or fixed by the experimenter, and one wishes to estimate the decay constant b. To what extent does the choice of values of x influence the estimate of b?

Figure 2 depicts this decay curve and offers several conceivable arrangements of values of x at which to measure y. The variance of the estimate of b [i.e., var(b)] which results from each of these designs is shown in Figure 2 as a multiple of the experimental error σ^2. Var(b) is seen to change by a factor of three over the possible designs shown here. Intuitively appealing designs that spread the observation over a wide range of x values yield estimates of b which are 50-100% more variable than the estimate found by taking all of the observations at x = 1/b. It can be shown that this is, in fact, the design which minimizes the variance of b.

Each observation that one takes contributes a specifiable amount of information toward estimating b. The amount of information conveyed by a given observation y_i depends entirely upon the value of x_i at which y_i is measured. This relationship is shown in Figure 3. In brief, for the exponential function, the most information is contained in an observation taken at x = 1/b. The farther from x = 1/b an observation is taken, the less information it contributes toward estimating b.

In general, minimum variance estimates of the parameters of any fairly simple model will be obtained by concentrating all of the observations at a number of specific points equal to the number of parameters in the model. In many cases (such as a straight line), one or more of the optimum observation points will be at extremes of the range of x. The exact points may be determined by optimization of the variance-covariance matrix (Draper and Smith 1981 discuss the variance-covariance matrix, and Box and Lucas 1959 deal with the optimization thereof), but it is not easy going. Alternatively, one may use an approximate method to find these points as outlined in Appendix 1 and discussed by Beck and Arnold (1977).

The obvious drawback of optimal designs is that they provide no means of checking the fit of one's model to the data. In practice, it is often desirable to sacrifice a certain amount of precision for the sake of ascertaining that one's model is adequate. It is still possible in such cases to evaluate the effect of the design matrix on parameter estimates. In general, if observations are spread out in the neighborhood of the optimal design points, a minimum of precision is sacrificed. For any given model, one may generate a sensitivity relationship such as Figure 3 and use this information to design one's experiments (or better still, use the covariance matrix approach).

Consider a second example, the familiar Michaelis-Menten model (or alternatively, the formally equivalent Monod function or Langmuir isotherm):

$$v = \frac{Vs}{K + s}$$

where v is the rate of reaction, s is the substrate concentration, and V and K are fitted constants (representing a maximum reaction velocity and a half-saturation constant, respectively). The most precise parameter estimates are obtained by taking half of the observations at s = K, and the other half at s >>K (i.e., as high as possible) (Currie 1982).

Figure 4 (taken from Currie 1982) appraises the effect of using geometric sequences (4A and 4B) and linear sequences (4C and 4D) of seven observations to estimate K and V. The linear sequences of observations s_i are defined by:

$$s_i = i\underline{a} \qquad \text{for } i = 1, 2, \ldots, 7$$

(for example, when $\underline{a} = 0.5$, the observations were taken at x values of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5) and the geometric sequences by:

$$s = \underline{a}\, 2^{i-1} \qquad \text{for } i = 1, 2, \ldots, 7$$

(for $\underline{a} = 0.5$, the observations were taken at 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0). In essence, $\underline{a}$ is a factor which serves to telescope the observations such that when $\underline{a}$ is small, the observations are concentrated nearer the origin, and when $\underline{a}$ is large, the observations are more spread out.

The lower panels of Figure 4 (4B and 4D) show the variance of estimates of K (i.e., K) as a function of the design matrix as characterized by $\underline{a}$. It is immediately evident that the linear sequences of observations in Figure 4D yield much higher variance estimates of K than do the geometric sequences in Figure 4B. Moreover, among each of these classes there are obvious minima in the variance of K which are obtained. The variance of parameter estimates using the best geometric sequence of observations is approximately twice as great as that obtained with the optimal design. However, the geometric spread of observations does allow one to verify the fit of the model while still producing reasonably precise parameter estimates. The catch, of course, is that one must have some preliminary estimate of K in order to design the experiment (similarly, for the exponential decay model, it is necessary to have some preliminary estimate of b). Given this information (usually from an exploratory experiment), one may then optimize the experimental design.

Not only is the precision of parameter estimates affected by the experimental design, the accuracy is affected as well. In Figure 4 panels A and C, the ratio of the estimated half-saturation constant (K) to its true value (K) is shown. This is a measure of bias: a ratio of 1.0 indicates an unbiased estimate of K, whereas deviations from 1.0 indicate less and less accurate estimates. It is evident that some sequences of observations yield badly biased estimates of K. The geometric sequences of observations (4A) are seen to yield estimates of K which are also more accurate than those from the linear sequences (4C).

The effect of experimental design on bias in parameter estimates is more difficult to determine than its effect on variance; in the present case, it was determined by Monte Carlo simulations, which are fairly involved. As a rule of thumb (which may not invariably be correct), it appears that the more precise parameter estimates also tend to be reasonably accurate.

INAPPROPRIATE FITTING TECHNIQUE

The most common means of fitting nonlinear models is to apply a transformation which linearizes the relationship and then to fit the data by the ordinary least-squares method. Generally speaking, this is inappropriate. Least-squares regression involves the assumption that the error in the dependent variable is normally distributed and of equal magnitude at all x. When a dependent variable is transformed, the error around that variable is also transformed and, depending upon the original distribution, the final error distribution will often be something very far from normal which varies with x.

Figure 5 shows the bias and variance of estimates of K in the Michealis-Menten model using the same data as in Figure 4. However, in the case of Figure 4, the untransformed data were fitted with a direct, nonlinear least-squares method (Bliss and James 1966; Hanson _et al_. 1967), whereas in Figure 5 the data were subjected to a Woolf linearizing transformation first, and were then fitted by ordinary linear least-squares. A comparison of the two figures reveals that the Woolf transformation produces parameter estimates which are strongly biased and which have very much higher variance than those found with the untransformed procedure (Figure 4). The Woolf transformation has been compared to other linearizing transformations of the Michaelis-Menten function, and it has been shown to be the _best_ of the possibilities (Dowd and Riggs 1965; Atkins and Nimmo 1975). As _a rule_, linearizing transformations will yield high biased, high variance parameter estimates, whereas direct untransformed fits of nonlinear functions will be much more reliable. Nonlinear fitting routines are now available in virtually all of the major main-frame statistical packages (e.g., NLIN in SAS, NONLINEAR in SPSS). Linearizing transformations may be appropriate in cases where a transformation is applied in order to normalize the error distribution. Obviously, the rationale here is that attention must be paid to the assumptions of one's fitting technique.

INAPPROPRIATE MODEL

A third common practice which leads to poor parameter estimates is that of fitting an inappropriate function for the sake of ease of computation. A frequent example is the case in which a straight line is fitted to something called "the initial linear portion" or the "first-order portion" of a nonlinear function. In most cases, theoretical considerations or examination of the residuals around the fitted function (e.g., Draper and Smith 1981) should be sufficient to show that there is no initial linear portion. Application of a linear model to an "approximately linear" curve will invariably lead to biased parameter estimates.

As an example, consider the data shown in Figure 6, which show the time course of incorporation of ^{32}P orthophosphate in a bacterial culture. The goal of this experiment was to estimate the rate of orthophosphate incorporation, which is known to decline as orthophosphate is depleted from solution. A very common procedure would be to fit a straight line to an initial "linear" portion of the curve and to take its slope as the rate of phosphorus incorporation. On cursory examination of the data, the assumption of initial linearity does not seem unreasonable. However, it is unclear exactly what portion of the curve should be considered as linear. The numbers in the upper left portion of the figure represent estimates of the slopes of straight lines fitted to progressively shorter initial segments of the curve. It can be seen that as the latest observations are sequentially eliminated from consideration, the estimated slope of the "initial linear portion" steadily increases. This is due to the fact that indeed there is no linear portion of the curve. As a result, the estimated rate of orthophosphate uptake is systematically underestimated, to an extent which depends upon the length of the period of observation.

It is evident that the initial rate of uptake is the biologically interesting parameter in this example. The solution to this fitting difficulty is to choose a function which eliminates the pattern in the residuals, and which is therefore insensitive to the duration of the experiment. A second degree polynomial was found to fulfill these criteria, and yielded the rate estimate in the lower right portion of the figure. This rate represents an unbiased estimate of the initial rate of orthophosphate uptake.

It is worth mentioning in passing that the straight line fits these data very well, as judged by the correlation coefficient or an F-test. Moreover, the second degree term of the polynomial fit was never significant according to a partial-F test. The second degree term was nonetheless retained in order to satisfy the assumption (of any regression method) that the error around the function is unbiased (i.e., that the expected value of the residuals is zero). Failure to satisfy this assumption is evidence of an inappropriate model, which causes parameter estimates to be biased.

SUMMARY

Fitting nonlinear models to experimental data poses a number of unique statistical problems in addition to those encountered in ordinary linear regression. A large number of biological studies involving nonlinear models make fundamental statistical errors which cause the parameter estimates and model predictions to be biased and highly variable. Many of these errors result from neglect of the basic assumptions of the fitting techniques used. Three especially common inappropriate procedures are: (1) neglect of the experimental design matrix (that set of values of the independent variable at which the experimenter determines to measure the dependent variable); (2) fitting data which have been transformed to remove nonlinearity without consideration of the effect on the error distribution; and (3) fitting the wrong function for the sake of ease of computation (e.g., fitting a straight line to the "initial linear portion" of a nonlinear function). Statistically appropriate alternatives to these practices are readily available.

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APPENDIX

Consider a model in which y is a function of x and a parameter θ:

$$y = f(x, \Theta)$$

The variance of the estimated parameter Θ is approximated by:

$$\mathrm{var}(\Theta) = \sigma^2 / \sum_{i=1}^{n} \Delta^2$$

where

$$\Delta = \frac{\delta f}{\delta \Theta}$$

and σ^2 is the error variance. Therefore Δ^2 evaluated at a given value of x is a measure of the amount of information that that observation contributes toward estimating the model parameter (Beck and Arnold 1977).

When a model involves two or more parameters, the situation becomes more complex since each observation contributes information to the estimation of every parameter. An exact solution to the problem of designing the experiment is given by Box and Lucas (1959; or Appendix I in Currie 1982). However, in most cases the method given above, carried out for each parameter in the model, gives an adequate indication of how to design an experiment.

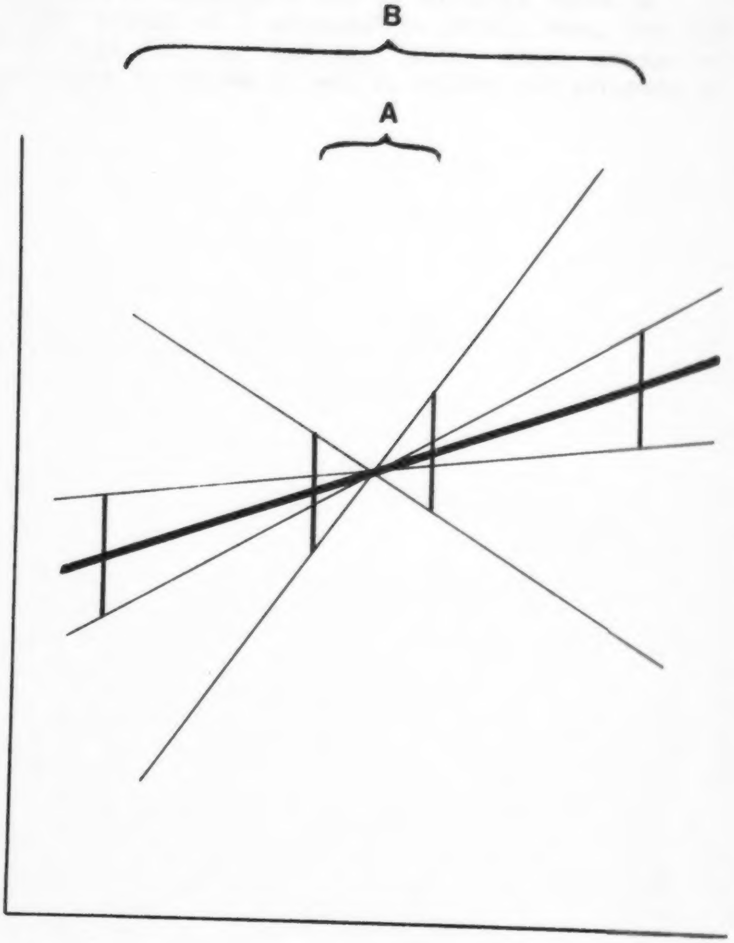


Figure 1. A schematic representation of the confidence interval around the estimate of the slope of a straight line, when observations are taken over a narrow range (A) versus a broader range (B).

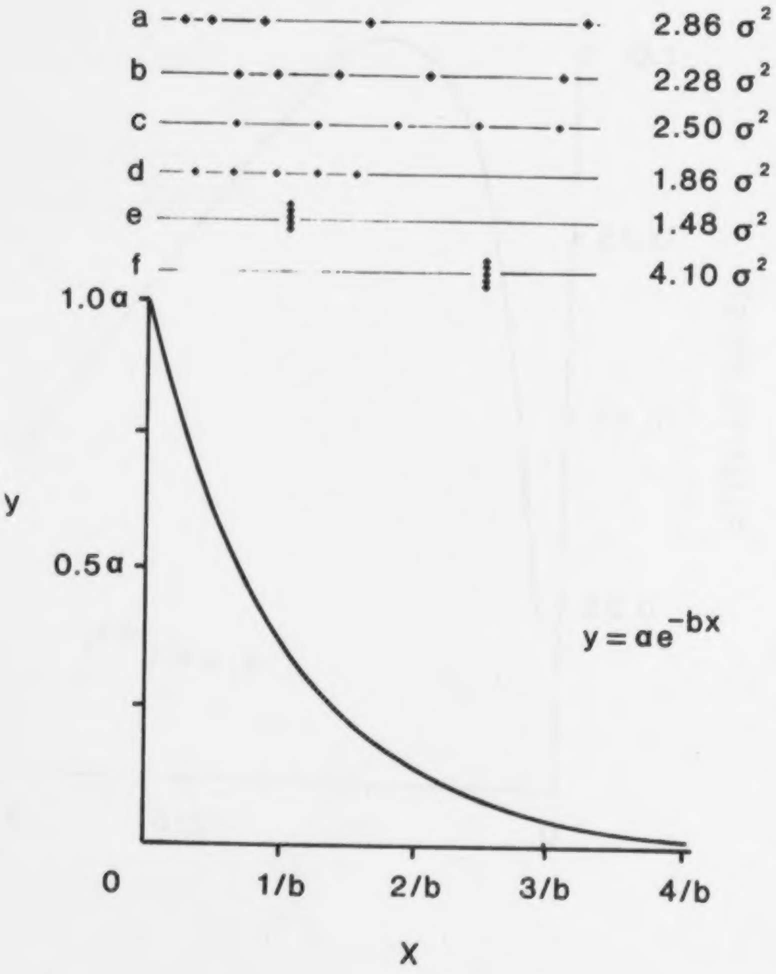


Figure 2. The effect of the experimental design on the precision of estimation of the decay constant b. Each array of diamonds (a-f) represents a series of five observations spread in various ways across the range of x. The resulting variance of the estimate of b is shown as a multiple of the error variance of σ^2.

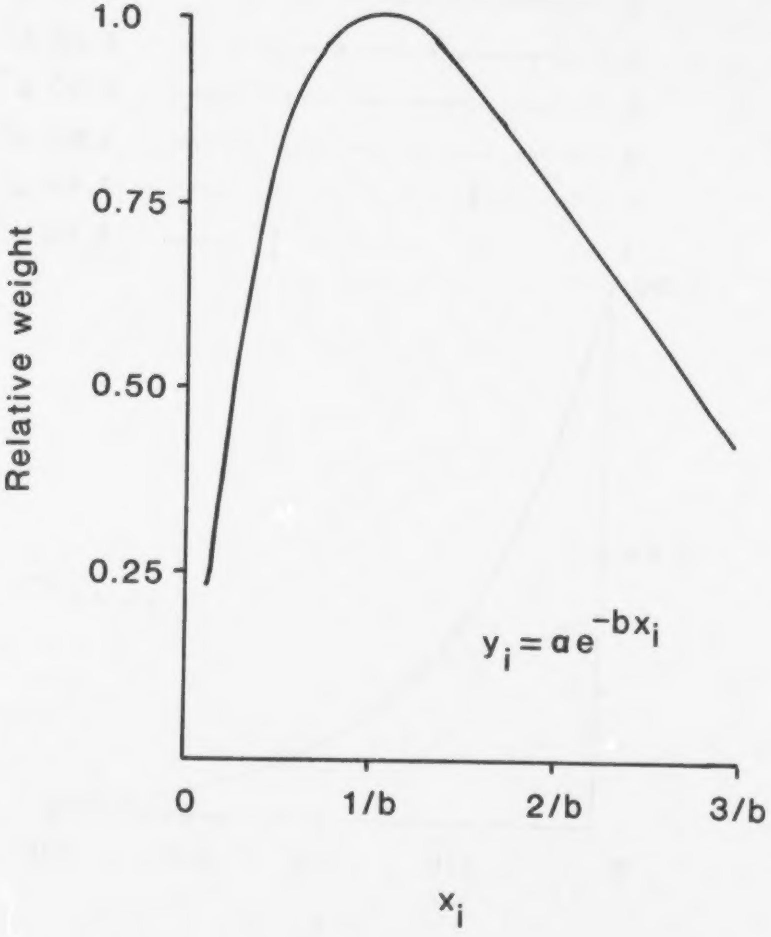


Figure 3. The relative amount of information which an observation y_i taken at a given x_i contribute to the estimate of b.

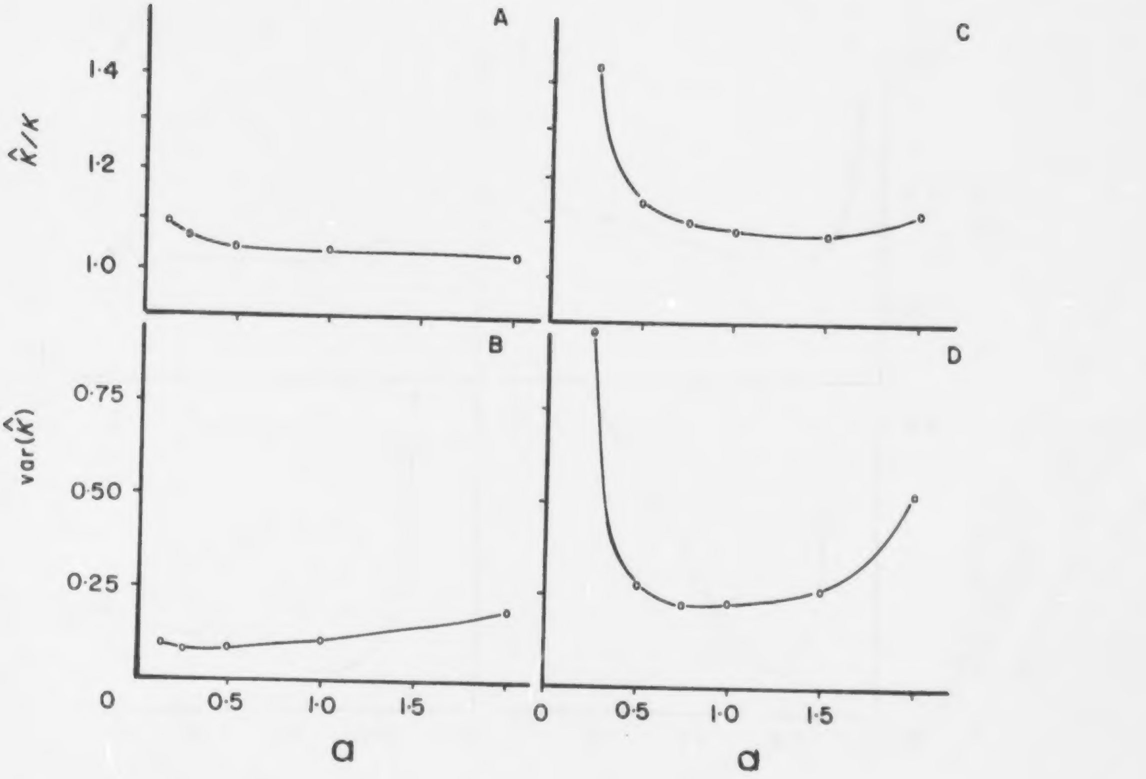


Figure 1. The bias ($K/\hat{K}$) and variance (var($\hat{K}$)) of estimates of the Michaelis-Menten K, as a function of the design matrix. Panels A and B represent the effect of geometric sequences of observations; panels C and D show the effect of linear sequences. In both cases, *a* serves to telescope out the observations such that then *a* is small, observations are concentrated near the origin, and when *a* is large, observations are more spread out. A nonlinear fitting technique was used. See text.

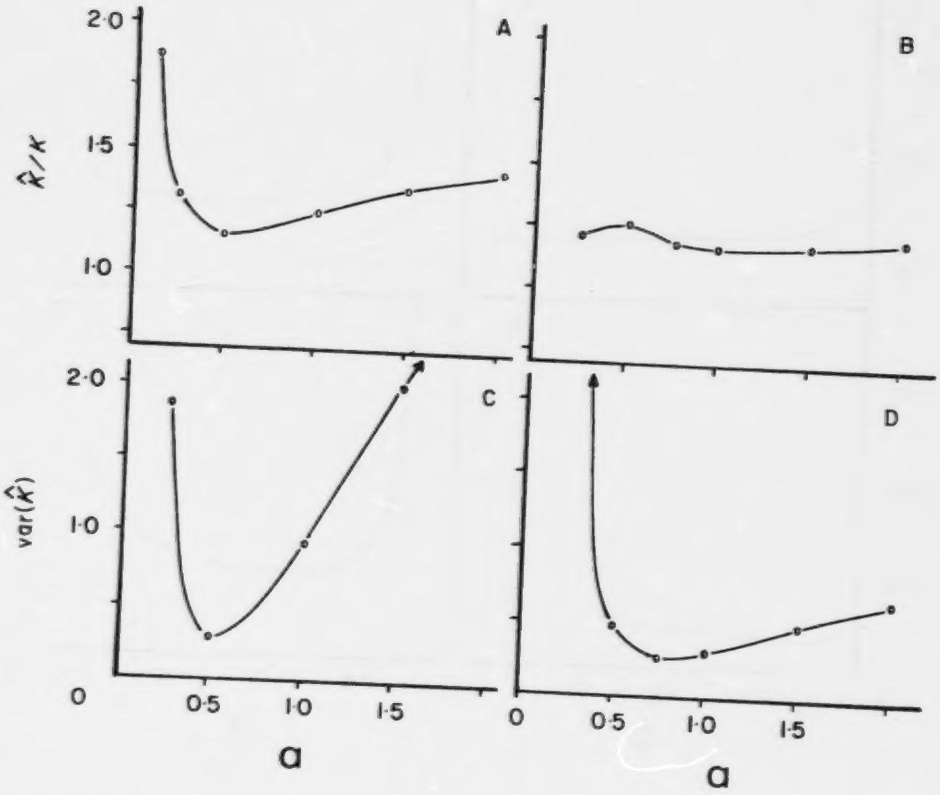


Figure 5. Same as Figure 4, using a linear fit to Woolf transformed data.

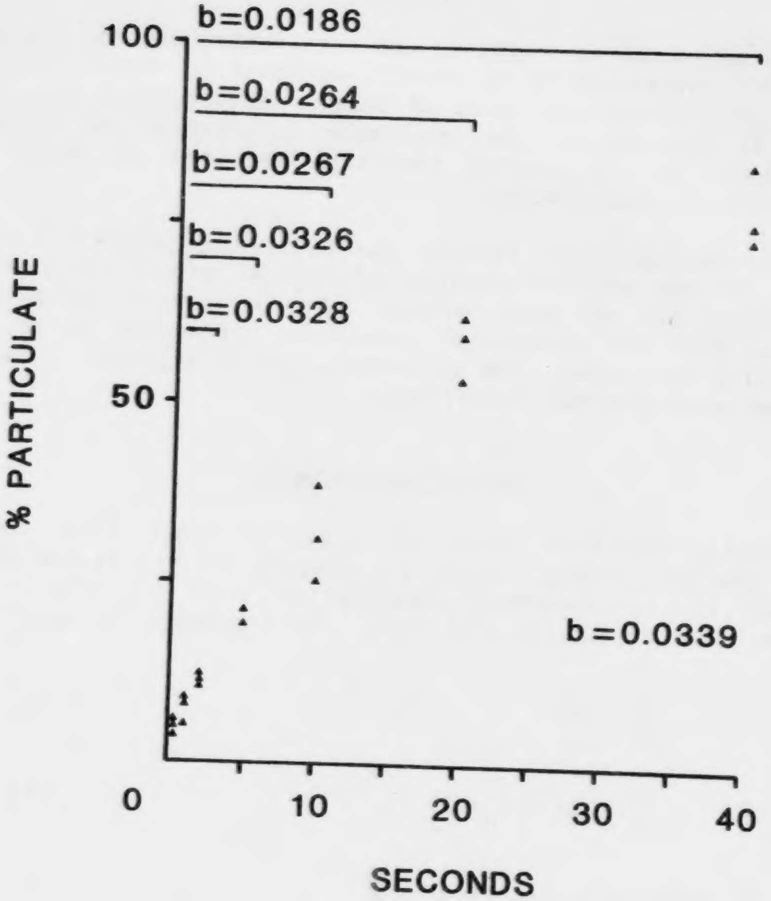


Figure 6. The estimated slopes of straight lines fitted to progressively shorter initial segments of a ^{32}P uptake curve. The initial slope as determine by a polynomial bit is b=0.0339.

EXPERIMENTAL REQUIREMENTS FOR MODELING DECAY CURVES

W. J. Maier

ABSTRACT

The objective in analyzing and correlating data on the decay of organic substrates is to develop mathematical models that can be used to characterize rates of decay. Mathematical models are needed to extrapolate the available information to different environments or to predict the time required to reach some predetermined concentration.

Mathematical curve fitting per se is not adequate for this purpose if the physical-chemical-biological significance of the coefficients has not been defined. A prerequisite for a useful model is that the equations' parameters correspond to the rate controlling reactions. The parameters can be adjusted to reflect different environmental conditions.

UNSTRUCTURED MODELS

Biochemically mediated decay of organics stems from the metabolic processes of the microflora. Rates are closely related to the concentration of organisms as an important description even if the microorganism concentrations remain constant with time. The framework for modeling is given by:

$$\frac{ds}{dt} = \frac{\mu x}{y} \qquad (1)$$

$$\frac{dx}{dt} = \mu x - k_d x \qquad (2)$$

where s = substrate concentration
x = concentration of microorganisms
μ = growth rate coefficient
y = yield coefficient
K_d = endogenous decay coefficient

For those situations where the decay rate is mediated by extracellular enzymes (E_e), it may be necessary to relate its concentration to x. The parameters μ and y must be defined in terms of the important process variables.

A number of alternate expressions have been proposed for μ and y as shown in Table 1. The most appropriate expression to use for a specific application depends on the range of substrate concentration of the decay curve and the presence of other competing organic substances.

TABLE 1
Alternate Expressions Proposed for the Growth Rate (μ) and yield (y) Coefficients

$\mu = \mu_m^*$	Exponential growth, unlimited
$\mu = \mu_m \frac{S}{K_s + S}$	Monod growth equation
$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{Ki}}$	Monod equation with substrate inhibition coefficient Ki
$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{Ki} + f(Sj)}$	Monod equation with terms to account for substrate inhibition and alternate substrate effects due to Sj substrates
$\mu = \sum_n \frac{Mm_{1n} S_n}{K_{s,n} + S_n + \frac{S_n^2}{K_{i,n}} + f(Sj)}$	Summation of growth rates represents concurrent utilization of alternate substrates i
$Y = \text{constant}$	
$Y = \frac{Y_T}{1 + kd/\mu}$	Yield coefficient is dependent on rates of endogenous decay and growth
$Y = f(T)$	Yield coefficient is temperature dependent

* μ_m is a function of temperature, pH, and ionic strength

The first two relationships in Table 1 for μ are widely used. The advantage of the simpler models is that the resulting equation can be linearized or solved explicitly. Cell mass concentration can be expressed in terms of equivalent substrate concentration, thereby eliminating it as a dependent variable. This allows calculating the numerical values of μ_m and K_s from measurements of substrate decay curves providing an independent measure of initial cell mass concentration is available.

Solution of the third equation in Table 1 which incorporates the effect of substrate inhibition requires making some simplifying assumptions. K_s can be evaluated from data at low concentration by neglecting the Ki term and conversely, Ki can be evaluated from high substrate concentration data. It is, however, necessary to factor in the time rate of change of cell mass concentration. Application of the last two relationships for growth rate expressions in Table 1 requires the concurrent analysis of several sets of decay curves to define the interaction effects, namely, inhibition by competing substrate and growth enhancement by concurrent substrate utilization. The equations cannot be solved explicitly. One approach is to correlate the data using the differential forms and cross correlate to describe the effects of alternate substrates. Cell mass concentration is a variable and must be factored into the analysis.

It is important to note that cell mass concentration (x) is a time dependent variable regardless of the relationship used to define μ. In some situations (when the initial cell mass concentration is large compared to the rate of additional cell production), it is acceptable to treat x as a constant, thereby simplifying the data analysis procedures. It is, however, necessary to obtain an independent measurement of the initial active cell mass concentration in order to characterize the substrate decay curve or to calculate the numerical value of μ from a decay curve.

In the more general situation where cell mass concentration changes with time, numerical integration of equations 1 and 2 can be used to simulate substrate decay curves.

Computerized subroutines for solving coupled differential equations are readily available. They can be used to predict decay curves from known values of μ_m, K_s, k_d, Y and they can be used to estimate the values of these parameters from measured substrate decay curves. Parameter estimation subroutines are available to calculate the best fit values for simple decay curves as well as for a sequence of decay curves starting with different initial concentration of cell mass or substrate.

Examples of the use of numerical analysis of laboratory test data are illustrated in the following figures.

Figure 1 shows decay curves of 2,4-D using acclimated inoculum at two different initial concentrations. The data (points) from both test runs were fitted by nesting the data. The kinetic parameters were estimated using a fitting subroutine.

Figure 2 shows 2,4-D decay curves and attendent increase in cell mass. Initial cell mass concentration was calculated using fitting subroutine.

Figure 3 shows an application of parameter estimation to fit 3,5-DCB decay curve. Lines illustrate the effects of changing the numerical values of the parameters μ_m, K_s, X_o. Best fit calculations become progressively more time consuming and less reliable for estimating multiple parameters.

Figure 4 shows measured decay curves of 2,4-D with and without addition of alternate substrate (nutrient broth). 2,4-D acclimated inoculum was added at time zero. Filtered inoculum had lower active cell mass and much lower protozoa concentrations.

Figure 5 shows measured decay of 2,4-D (same as Figure 4a-4b) and model calculations of substrate decay curves. Model calculations incorporate effects of substrate inhibition, substrate interaction, and concurrent substrate utilization.

SEMI-STRUCTURED MODELS

A major drawback of unstructured models is that they are limited to describing the kinetics of balanced growth, sometimes referred to as steady state growth kinetics. Time dependent growth phenomena have been described in a variety of situations. The most obvious cases occur when a new substrate that requires acclimation of enzyme systems is suddenly made available.

Recent advances in modeling time dependent growth phenomena are largely based on advances in understanding of the regulatory processes that control enzyme production and activity. Recognition that some catabolic enzymes are constitutive, e.g., formed at constant rates and amounts, while others are inducible, e.g., affected by concentration of specific substrates or metabolites, has been particularly helpful in explaining and modeling the growth behavior of unacclimated cultures. Changes in growth rate are seen to be the result of changes in enzyme activity levels; different enzyme controlled reactions can become the rate limiting step in the overall sequence of growth related metabolism; hence, the need for a structured model. However, it has also become evident that complete stepwise modeling of growth processes is extremely complicated and probably not feasible for routine use. As a result, model development has focused on semi-structured models as a compromise between rigor and practicality. Semi-structured models have found favor because they can be used to describe time-dependent growth phenomena without exceeding practical limits on the number of rate coefficients, equilibrium constants, and rate equations needed to describe the system.

One version of a two-enzyme model has been described to illustrate its application for analyzing and correlating lag phase behavior. The model considers two rate controlling steps, namely, substrate transport by permeases followed by intracellular metabolism. The data represent the observed lag phase when a 2,4-D acclimated culture is challenged with mixtures of 2,4-D and glucose. The model assumes that transport and conversion of 2,4-D and glucose are mediated by their respective permease enzyme systems E_{11} and E_{12}; P_1 and P_2 are internally available intermediates and are used for growth and energy metabolism by their respective enzyme systems, E_{21} and E_{22}. The presence of P_1 and P_2 can affect permease enzyme production or activity; repression of permease by the other intermediate was assumed. The model is schematized in Table 2.

The corresponding mathematical formulations for calculating the time-concentration changes of the two substrates (S_1 and S_2), their corresponding intracellular products (P_1 and P_2), enzymes (E_{11}, E_{12}, E_{21}, E_{22}) and overall cell mass formation (X) subject to material balance considerations are summarized in Table 3a; the symbols are defined in Table 3b.

Published information on kinetic coefficients for transport enzymes and overall catabolic enzyme systems was used. Test data using single substrates were used to calculate coefficients for the acclimated culture. The system of differential equations was solved by numerical integration using a Runge-Kutta subroutine available through the University of Minnesota Computer Center. The initial values of substrate and cell mass are measured.

TABLE 2

2-enzyme model, concurrent substrate utilization
Version II - Different intracellular intermediates

Case I - Glucose permease repressed by 2,4-D intermediate
2,4-D permease repressed by glucose intermediate

Uptake:

$$S_1 \xrightarrow{E_{11}} P_1$$

$$S_2 \xrightarrow{E_{12}} P_2$$

Growth

$$P_1 \xrightarrow{E_{21}} X$$

$$P_2 \xrightarrow{E_{22}} X$$

The model:

Uptake:

$$E_{11} + S_1 \xleftrightarrow[K_{21}]{k_{11}} E_{11}S_1 \xrightarrow{k_{31}} E_{11} + P_1$$

$$E_{12} + S_2 \xleftrightarrow[k_{22}]{k_{12}} E_{12}S_2 \xrightarrow{k_{32}} E_{12} + P_2$$

Growth:

$$E_{21} + P_1 \xleftrightarrow[k_{21}]{k'_{11}} E_{21}P_1 \xrightarrow{k'_{31}} E_{21} + X$$

$$E_{22} + P_2 \xleftrightarrow[K'_{22}]{k'_{12}} E_{22}P_2 \xrightarrow{k'_{32}} E_{22} + X$$

TABLE 3a
Rate Equations

$$\frac{dS_1}{dt} = -k_{31} \frac{a_1}{b_1} \frac{[E_{11,TOT}][S_1]}{k_{S_1} + S_1}$$

$$\frac{dS_2}{dt} = -k_{32} \frac{a_2}{b_2} \frac{[E_{12,TOT}][S_2]}{k_{S_2} + S_2}$$

$$\frac{dP_1}{dt} = K_{31} \frac{e_1}{b_1} \frac{[E_{11,TOT}][S_1]}{k_{S_1} + S_1} - k_{31} \frac{e_1}{f_1} \frac{[E_{21,TOT}][P_1]}{k_{P_1} vX + P_1}$$

$$\frac{dP_2}{dt} = k_{32} \frac{e_2}{b_2} \frac{[E_{12,TOT}][S_2]}{k_{S_2} + S_2} - k'_{32} \frac{e_2}{f_2} \frac{[E_{22,TOT}][P_2]}{k_{P_2} vX + P_2}$$

$$\frac{dX}{dt} = k'_{31} y_{P_1} \frac{e_1}{f_1} \frac{[E_{21,TOT}][P_1]}{k_{P_1} vX + P_1} + k'_{32} y_{P_2} \frac{e_2}{f_2} \frac{[E_{22,TOT}][P_2]}{k_{P_2} vX + P_2} - k_d X$$

$$\frac{dE_{11,TOT}}{dt} = \frac{c_1}{d_1 + l_1 P_2^{m}\,1} \frac{dX}{dt}$$

$$\frac{dE_{12,TOT}}{dt} = \frac{c_2}{d_2 + l_2 P_1^{m_2}} \frac{dX}{dt}$$

$$\frac{dE_{21,TOT}}{dt} = \beta_1 \frac{dX}{dt}$$

$$\frac{dE_{22,TOT}}{dt} = \beta_2 \frac{dX}{dt}$$

TABLE 3b
Explanations and Symbols

S_1: glucose concentration, mg/l

S_2: 2,4-D concentration, mg/l

P_1: glucose intracellular intermediate, mg/l

P_2: 2,4-D intracellular intermediate, mg/l

X: biomass, mg/l

$E_{11,TOT}$: total glucose transport enzyme, mg/l

$E_{12,TOT}$: total 2,4-D transport enzyme, mg/l

$E_{21,TOT}$: total glucose intermediate metabolic enzyme, mg/l

$E_{22,TOT}$: total 2,4-D intermediate metabolic enzyme, mg/l

k_{S_i}: Michaelis constant for S_i, $i=1,2$; mg/l

k_{P_i}: Michaelis constant for P_i, $i=1,2$; mg/l

K_{3i}, k'_{3i}: enzyme rate constants, $i=1,2$; hrs^{-1}

k_d: biomass death rate coefficient, hrs^{-1}

y_{P_i}: yield coefficient, mg biomass/mg P_1; $i=1,2$

a_i, b_i, e_i, f_i: molecular weights for S_i, E_{1i}, P_i, E_{2i}

v: specific volume of biomaterial, l/mg

MULTIPLE COMPONENT MODELS AND STEPWISE DEGRADATION OF COMPLEX SUBSTRATES

Stepwise degradation of complex substrates can involve one or more species of microorganisms acting concurrently. Oxidation of ammonia to nitrite and nitrate by nitrosommas is a classical example of a two species process. Measurements of monochlorobiphenyl degradation show evidence of sequential biodegration.

Models that explicitly simulate substrate utilization with formation and utilization of intermediates by independently acting microorganism populations have been defined. These models have the same form as the single substrate models described in previous sections. The only difference is that the inventory of each microorganism population is calculated using a series of coupled differential equations. The availability of the second step substrate (product from first step) is dependent on the sites of utilization of both species.

The principal criterion for determining whether it is necessary to use a multicomponent model is the formation and accumulation of product intermediates. If the measured concentrations of intermediates are negligibly small, it is reasonable to use a single culture model. If intermediate products tend to accumulate at a slow rate, the use of a multicomponent model is recommended.

PROTOZOA PREDATION EFFECTS

The role of protozoa in controlling bacterial populations has been recognized and documented by a number of investigators in wastewater treatment. For example, the rate of 2,4-D utilization with 3 micron filtered inoculum resulted in higher maximum rates of substrate removal. This has been ascribed to the effect of removing protozoa thereby reducing predation effects. Figure 7 illustrates the phenomenology for 2,4-D utilization and attendent population dynamics of bacteria and protozoa.

Protozoa predation has usually been modeled indirectly by introducing the endogenous decay term kd shown in equation 2. This approach appears to be satisfactory for analyzing continuous flow reactor systems. However, it is not adequate for simulating the phenomenology described in Figure 7 which represents batch test data and is typical of shock loading situations.

Explicit modeling of protozoa predation has been defined. A prey predator model has been used to simulate the data in Figure 7. Kinetic coefficients that describe the growth of protozoa and bacterial decay were calculated and give a reasonable fit of the data.

The methods for measuring microbial concentrations (bacteria and protozoa) are time consuming and relatively few studies have been published. As a result, it has not been possible to validate prey predator models adequately. Additional test data that are representative of conditions in natural bodies of water are needed to define the potential importance of protozoa and other bacterial dieoff mechanisms that could affect population dynamics.

SUMMARY

It is recommended that the measurement and modeling of substrate decay phenomena be broadened to include characterization of the accompanying changes in active cell mass or enzyme concentrations. Quantitative descriptions of cell mass are essential in order to obtain precise information on rates and allow extrapolation of data to other environments.

The form of the rate equation that is best suited for analyzing test data depends on the complexity of the environmental system to be characterized. Substrate inhibition effects must be incorporated at elevated concentrations; the critical concentrations vary considerably from 100+ milligrams per liter to 300+ micrograms per liter for 2,4-D and PCP* respectively. The effects of alternate substrates can be an important factor in regard to the utilization of the target substrate and in enhancing the proliferation of active cells. The effects of alternate substrates should be considered at all concentration levels. This also implies that the decay curves of alternate substrate should be measured concurrently with target substrate decay rates.

It is further recommended that semi-structured models be used for analyzing the correlating data that represent transients or acclimation periods. The greater complexity of these models is compensated for by the advantages that accrue from more detailed mechanistic descriptions of the rate controlling steps. For example, the semi-structured modeling approach can be used to simulate the effects of specific enzyme systems thereby giving insight into mechanisms. Simulation studies can also be used to specify enzyme activity measurements that are needed to describe the transients or acclimation process.

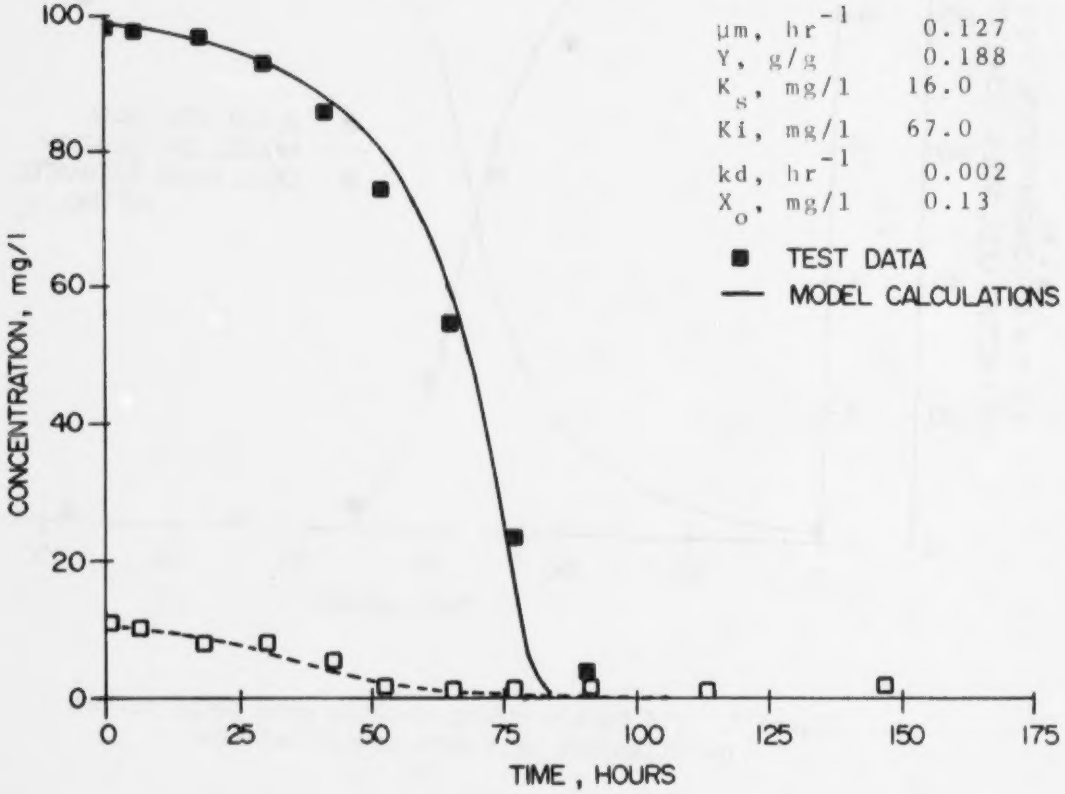


Figure 1. 2,4 Dichlorophenoxyacetic acid decay curves.

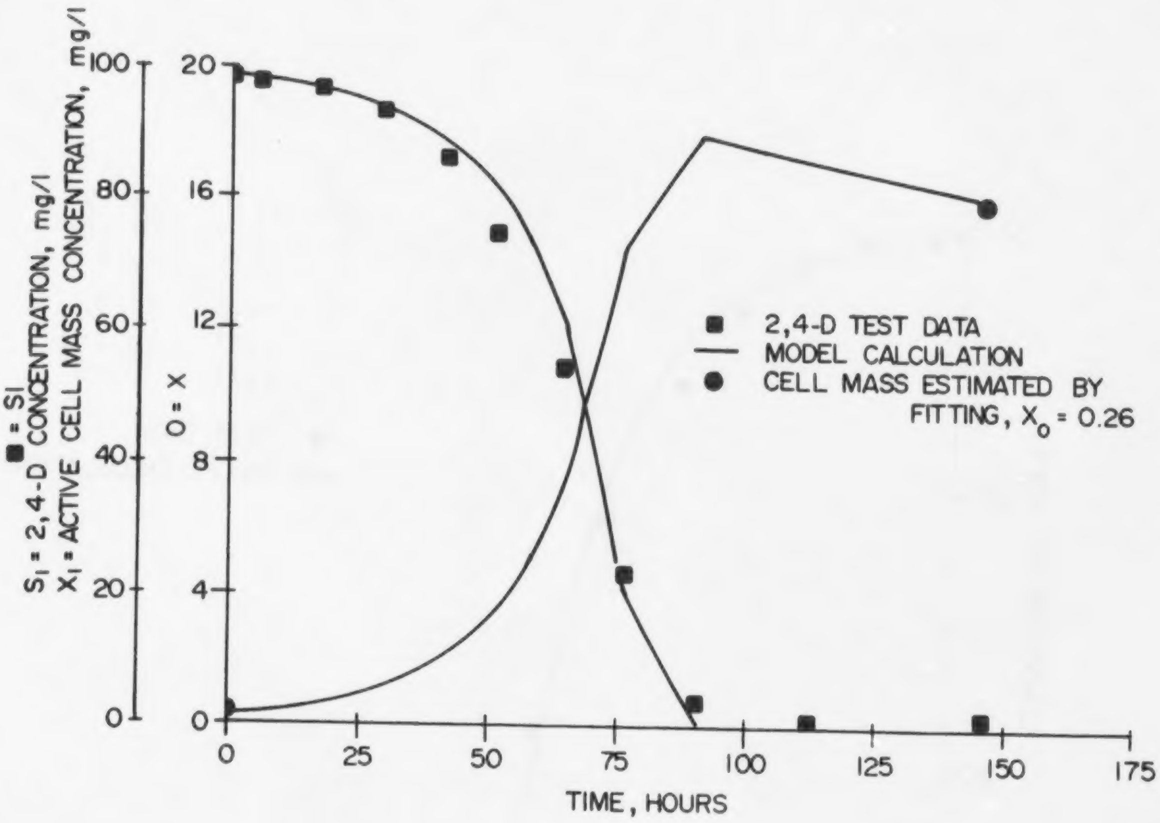


Figure 2. 2,4 Dichlorophenoxyacetic acid decay curves and attendent cell mass concentrations.

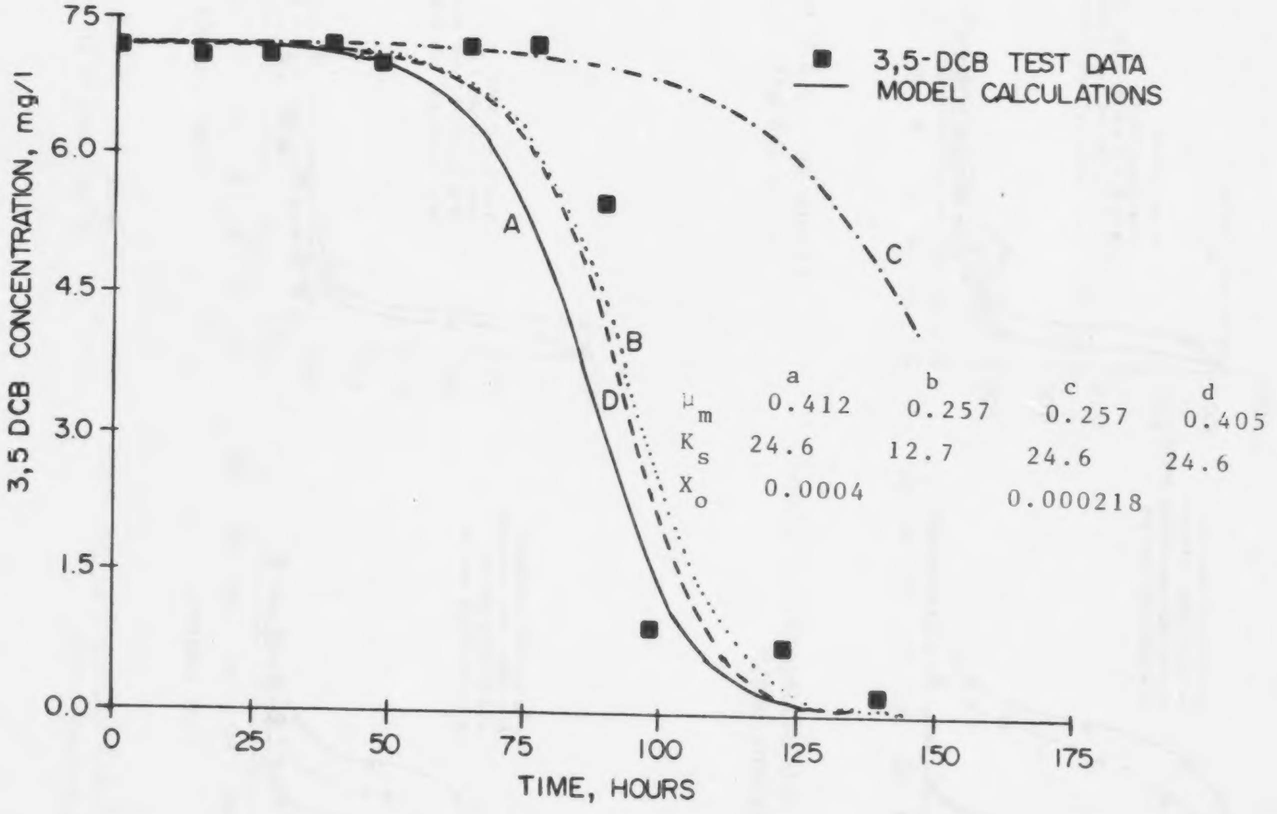


Figure 3. 3,5 Dichlorobenzoate decay curves.

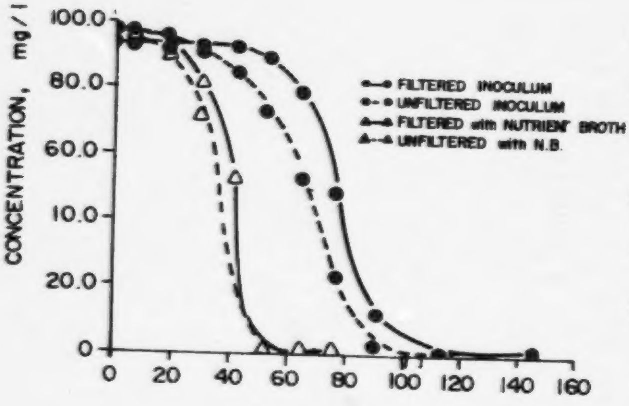


Figure 4a. 2,4-D biodegradation alone and with nutrient broth.

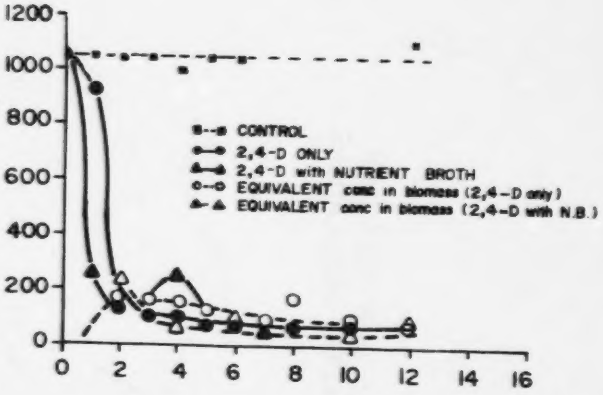


Figure 4c. 2,4-D biodegradation alone and with nutrient broth.

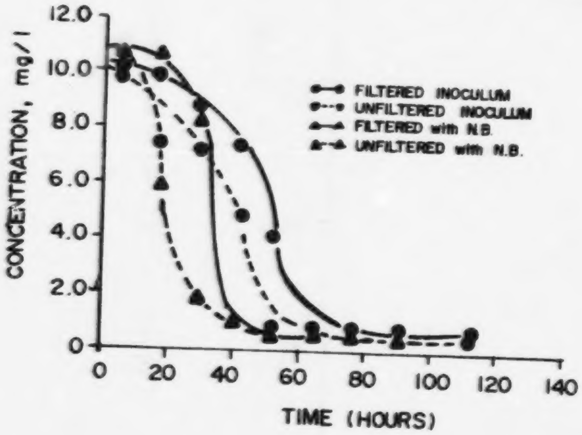


Figure 4b. 2,4-D biodegradation alone and with nutrient broth.

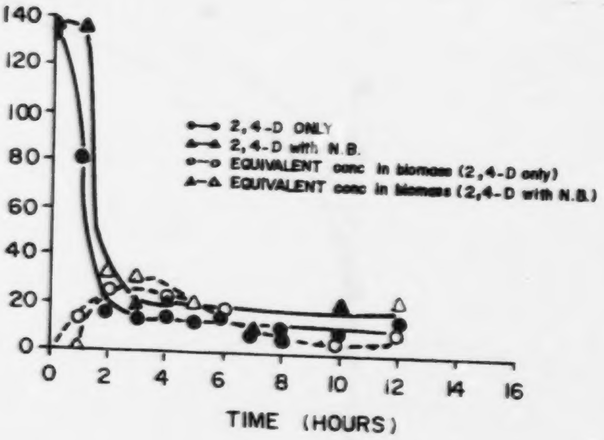
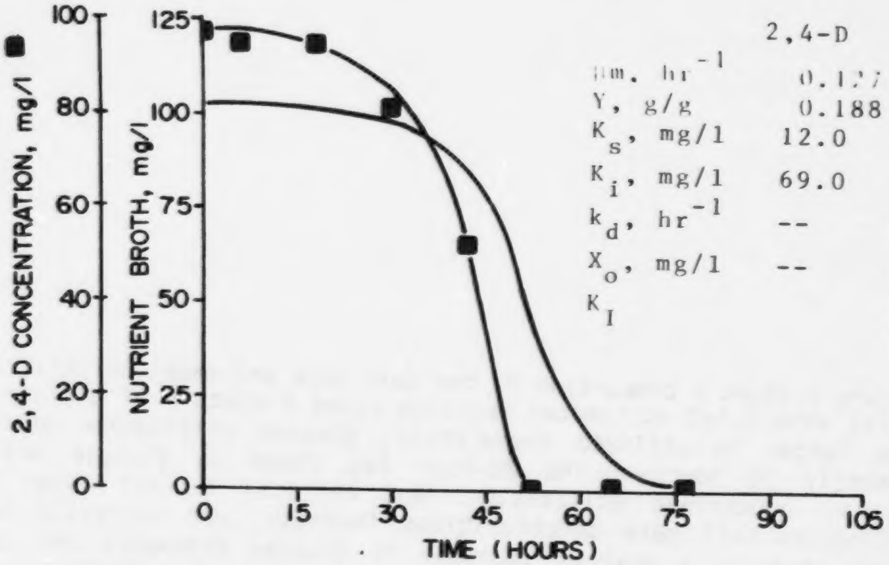
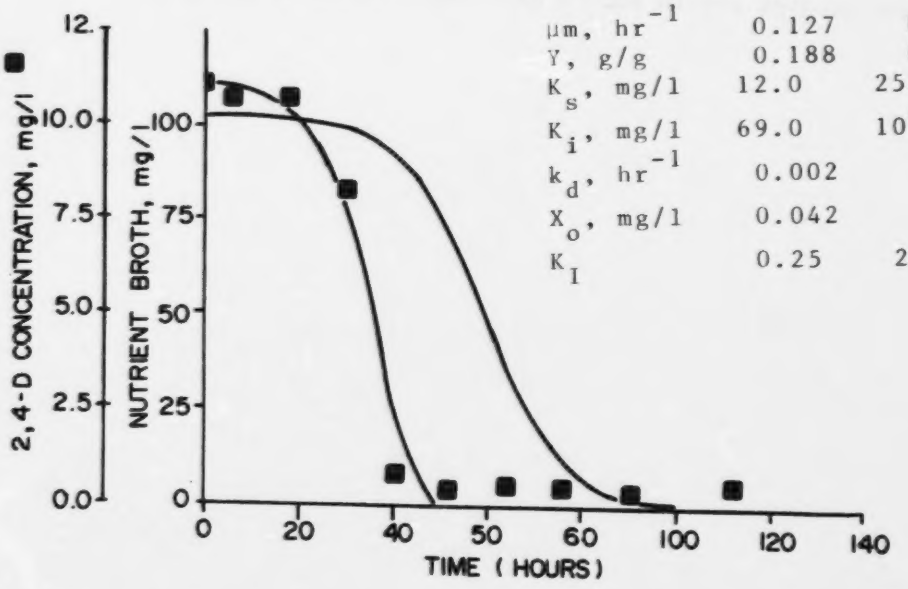


Figure 4d. 2,4-D biodegradation alone and with nutrient broth.



	2,4-D		N.B.
μm, hr^{-1}	0.127		0.32
Y, g/g	0.188		0.4
K_s, mg/1	12.0		257.0
K_i, mg/1	69.0		10^4.
k_d, hr^{-1}	--	0.002	--
X_o, mg/1	--	0.3	--
K_I		0.25	25.0

Figure 5b. Concurrent Utilization of 2,4-D and Nutrient Broth



μm, hr^{-1}	0.127	0.32
Y, g/g	0.188	0.4
K_s, mg/1	12.0	257.0
K_i, mg/1	69.0	10^4.
k_d, hr^{-1}	0.002	
X_o, mg/1	0.042	
K_I	0.25	25.0

Figure 5c.. Concurrent Utilization of 2,4-D and Nutrient Broth

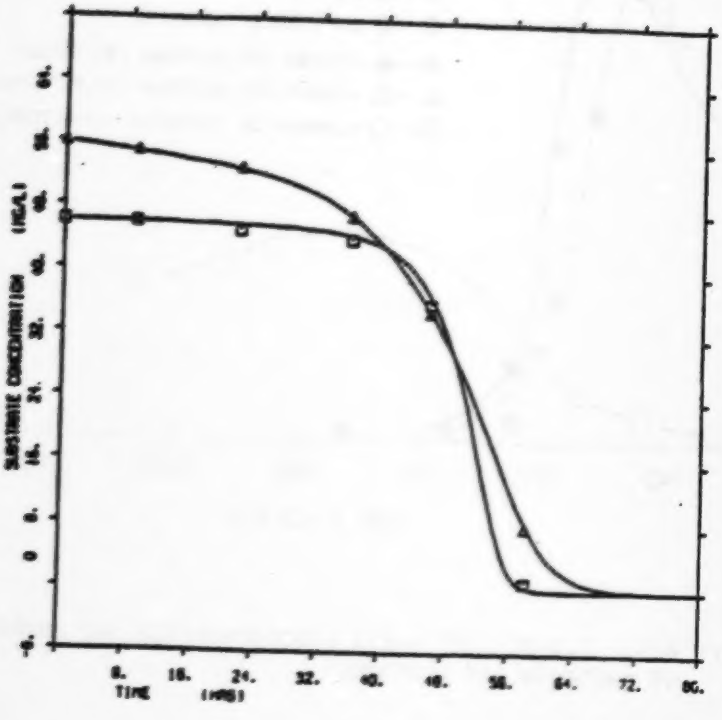
Figure 6 shows a comparison of the test data and model calculations for a batch test with 2,4-D acclimated inoculum using a mixture of glucose and 2,4-D. The latter is utilized immediately; glucose utilization starts after approximately 20 hours. The 20-hour lag phase in glucose utilization, followed by concurrent utilization, and enhanced overall rates of 2,4-D utilization as cell mass concentrations increase, was correctly simulated. The model predicts a gradual increase in glucose transport and catabolism until a balanced enzyme activity distribution that describes concurrent substrate utilization is achieved. The latter condition represents pseudo balanced growth with concurrent utilization of both substrates. The excellent agreement between data and model simulation is noteworthy because the findings of these studies are in accord with basic research using defined systems.

Figure 6

Model Calculations, Version II, Case I

Δ : Experimental data on glucose

□ : Experimental data on 2,4-D



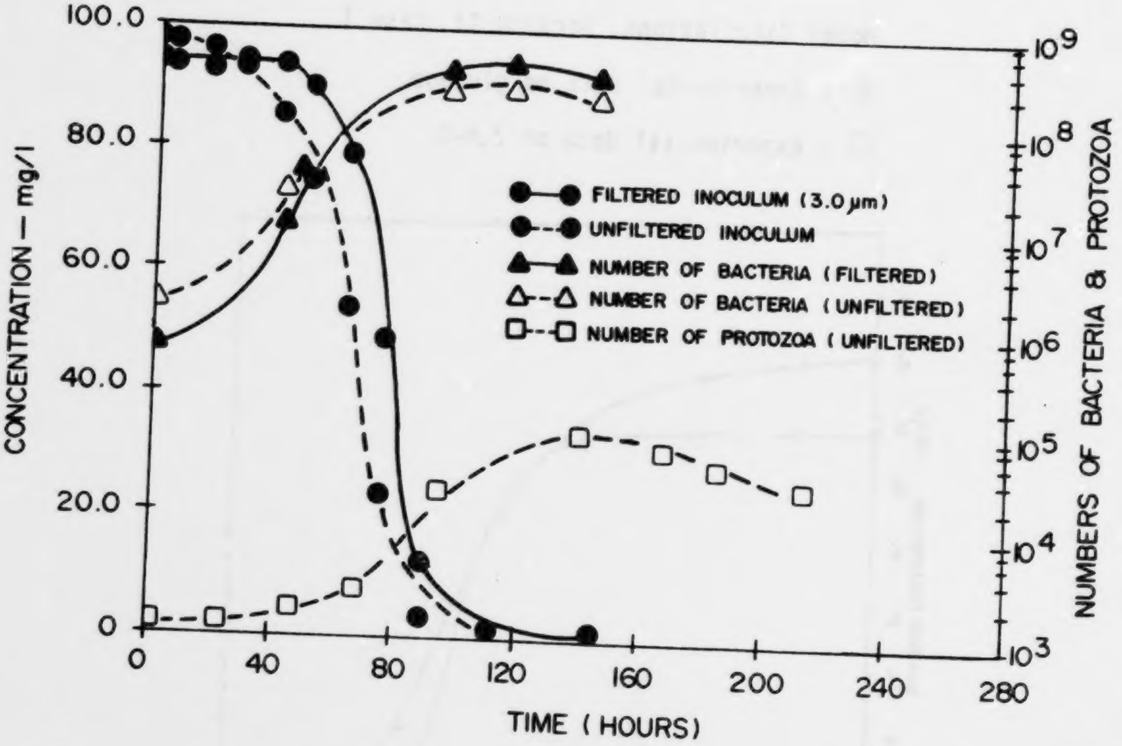


Figure 6.5. 2,4-D(= 100 mg/l) Biodegradation and numbers of bacteria and protozoa.

Our recommendations on statistical techniques to be applied to future studies in biodegradation must be divided into two sets. One set of recommendations applies to those individuals (Group I) who feel that estimating biodegradation rate coefficients to within an order of magnitude of their correct value is acceptable. This acceptance largely results from the level of noise (error) in the data from which these parameters are estimated. A different set of recommendations applies to the second group (Group II), which is interested in more precise parameter estimates and mechanistic model-building. Presumably, individuals in the latter group work with data several-fold more precise than the data to which the first group has access.

RECOMMENDATIONS FROM GROUP I

1. Never propose a model that is more complex than your data will support. This becomes especially important when the level of error is high (e.g., when the standard deviation of replicate measurements of the dependent variable exceeds 10% of the average value of the dependent variable).

2. If more complex models are to be used, then the addition of higher-order terms (e.g., nonlinear terms) should be tested for by using an F-test.

3. Use nonlinear parameter estimation (NPE) techniques to estimate microbial rate coefficients, when possible, but recognize that estimating these coefficients by using linearized forms of nonlinear models probably will not produce disastrous results. Fitting the data directly to a nonlinear model, rather than to a linearized form, can buy the investigator protection even when the level of noise is high.

RECOMMENDATIONS FROM GROUP II

1. Given a data set, propose several competing models to represent the underlying biological process. Differences among these models may be initially assessed via simulation. The simulated decay curves may subsequently be used to aid the design of an experimental program.

2. Estimate all parameters for the proposed models via NPE. The standard errors of the parameter should also be approximated. Explicitly state the updating method used to fit the data directly to the nonlinear models. No single method for estimating the parameters of nonlinear models is best and there are several algorithms available. The most commonly used NPE method -- typically available on main-frame computer systems -- is the Levenberg Marquardt algorithm. The Gaussian method is another popular NPE technique.

3. Select the "best" of the proposed models by using an F-test or similar model-building test.

4. Once the appropriate model has been selected, additional experiments may be run to obtain even more precise estimates of the microbial rate coefficients. In these cases, optimal design criteria should be used to select the range of measurement of the dependent variable. This will ensure that the variance of the estimated parameters is minimized.

   As a simple example, assume that the first-order model has been found to best represent a given data set by using an F-test. Now additional experiments are to be run with the goal of obtaining more precise estimates of K, the first-order decay coefficient. The optimal design for these experiments is to center the measurements of the dependent variable (presumably, substrate concentration) on the point at which the dependent variable is 37% of its starting value. Of course, data obtained for the optimally designed experiments should be analyzed by using an NPE method, again stating the technique of choice.

5. Robust regression (e.g., bi-weight regression) may be used to minimize the influence of outliers when data are fitted to a particular nonlinear model, but only when it is known a priori that the data are best represented by the chosen nonlinear model.

GENERAL REFERENCES

The following general references may be used for details on fitting nonlinear models to data. Further, some of these references provide information on optimally designing experiments for parameter estimation. The textbook by Beck and Arnold pays a great deal of attention to the statistical assumptions necessary for the correct application of least-squares analysis.

1. Beck and Arnold (1977). Parameter Estimation in Engineering and Science. John Wiley & Sons, Inc.
2. Draper and Smith (1981). Applied Regression Analysis, 2nd ed. John Wiley & Sons, Inc.
3. Wonnacott and Wonnacott (1971). Econometrics. John Wiley & Sons, Inc.

THE "SECOND ORDER" APPROACH:
ASSUMPTIONS, LIMITATIONS, AND RESEARCH NEEDS

Objective: To review the conceptual framework and assumptions associated with deriving biodegradation rates from decay curves which appear first order with respect to chemical concentration and whose slopes are linearly related to catalyst concentration. To develop a strategy for future research.

Many chemicals appear to degrade exponentially when exposed to inocula taken directly from the field. Plotting the logarithm of the chemical concentration against time produces a straight line. The slope of this line is generally considered to be a rate constant which is first order with respect to chemical concentration if the catalyst concentration remains constant during the experiment and the chemical concentration is below the half saturation constant (K_s). Moreover, it is assumed that differences in the first order rates from one experiment to another are due to differences in the catalyst concentration. How valid are these assumptions? Do they need further experimental verification and how? Are there special cases where they apply? What range of chemical concentrations needs to be tested? Are catalyst concentrations being determined properly and effectively? Are biodegradation rate constants for any particular chemical the same, regardless of the source or composition of the biological catalysts? What experimental evidence is needed to substantiate or refute this concept? What alternative mathematical expressions can be used or developed to describe exponential decay?

Panel Members: Bob Boethling (Chairperson), U.S. EPA
John Rogers, Battelle Northwest Laboratory
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THE " SECOND - ORDER " MODEL - AN OVERVIEW

Robert Boethling

THEORY

Kinetic models to predict rates of biodegradation of chemical substances have generally relied upon the empirically derived Monod equation (Monod 1949). According to Monod:

$$\frac{d[B]}{dt} \quad = \quad \frac{U_{max}\,[B]\,[S]}{K_s + [S]} \qquad (1)$$

where [B] is the microbial "concentration," V_{max} the maximum rate of growth of the population, [S] is the concentration of the growth - limiting substrate, and K_s is [S] when the growth rate is 1/2 V_{max}. It is evident that the Monod equation is an expression of the rate of growth of the population, d[B]/dt, in terms of population size and substrate concentration. To derive an expression for the rate of biodegradation of a substrate S, equation (1) may be modified by addition of a yield coefficient,

$$Y = d[B]/d[S] \qquad (2)$$

which describes the efficiency of conversion of substrate into microbial biomass. By multiplying equation (1) by 1/Y adding a negative sign to convert equation (1) to an expression for loss of S, one obtains equation (3).

$$\frac{d[B]}{dt} \quad X \quad \frac{d[S]}{d[B]} \qquad\qquad \frac{1}{Y}\;\frac{U_{max}\,[B]\,[S]}{K_s + [S]}$$

$$\frac{-d[S]}{dt} \qquad\qquad \frac{U_{max}\,[B][S]}{Y\ (K_s + [S]} \qquad (3)$$

The expression -d[S]/dt is equivalent to the rate of disappearance of the growth - limiting substrate.

To make use of equation (3), one must assume that the rate of population growth is limited by the substrate S over the expected range of [S]. This is not likely in environmental situations, however, because many other compounds that can serve as sources of carbon and energy are commonly available.

The difficulty of studying bacterial growth kinetics at environmentally relevant concentrations of S, where S is truly growth - limiting, is amply illustrated by the work of Shehata and Marr (1971) with pure cultures of Escherichia coli. To remove traces of utilizable carbon that would have precluded growth limitation by the added substrate glucose at parts - per - billion (ppb) levels of that sugar, procedures such as distillation of water from permanganate solution and exhaustive washing of glassware with chromi acid were required.

A closely related problem arises from consideration of the yield coeeficient Y. When substrate is growth limiting, Y approaches zero as substrate is utilized for growth and energy, and [S] approaches zero. Although not obvious from equation (2), this phenomenon is well documented and is a reflection of the diversion of substrate metabolism from growth to the need for "energy of maintenance" under conditions of carbon and energy needs may be provided by metabolism of those substrates. The practical outcome is that the "correct" value of Y to use in equation (3) cannot easily be determined.

If one is willing to disregard these theoretical difficulties, equation (3) can still be used to derive first-and second-order rate expressions for biodegradation. If the concentration of substrate, [S], is much greater than K_s, $K_s + [S]$ approximates [S] and equation (3) reduces to:

$$\frac{-d[S]}{dt} = \frac{U_{max}}{Y}[B] \qquad (4)$$

In this case the rate of substrate disappearance is proportional to the microbial concentration, [B], but not to substrate concenration. This is the usual situation in laboratory studies of microbial growth in pure culture, where substrate is plentiful. Environmentally, we are usually more concerned with situations in which [S] is lower. If [S] is much lower than K_s, $K_s + [S]$ approximates K_s and equation (3) reduces to:

$$\frac{-d[S]}{dt} = \frac{U_{max}[B]\ [S]}{YK_s} \qquad (5)$$

Equation (4) is a first-order kinetic expression because only one term is present as an independent variable. Equation (5) is a second-order expression because substrate disappearance is dependent upon two variables, [B] and [S].

The kinetic constants U_{max}, and K_s and yield coefficient Y (assumed to be constant) may be expressed as a single constant K:

$$K = \frac{U_{max}}{YK_s} \qquad (6)$$

Equation (5) then becomes:

$$\frac{-d[S]}{dt} = K\,[B]\,[S] \qquad (7)$$

Equation (7) is the second-order rate expression used EPA's Athens laboratory, and K is the second-order rate constant. Because of experimental difficulties in measuring U_{max}, Y and K_s for many organic chemicals, K is usually determined from equation (7) rather than equation (6). Rearrangement and intergration (assuming [B] is constant) of equation (7) yields equation (8):

$$\ln [S] = -K[B]t + \ln [S_0] \qquad (8)$$

where $[S_0]$ is the concentration of substrate at the start of the experiment. A plot of ln [S] vs. t should yield a straight line having a slope of -K[B] and a y intercept of ln $[S_0]$. The second-order rate constant, K, is obtained from the positive slope of equation (8) by simply dividing by the microbial concentration, [B]. The positive slope of equation (8), K[B], is also referred to as the pseudo first-order rate constant. It is clear from equation (8) that only disappearance of parent compound and microbial concentraion must be determined experimentally.

Unfortunately, the assumption that [S] is typically much lower than K_s, necessary to derive equation (7), may be a major theoretical stumbling block. Values of K_s for naturally occurring organic compounds such as sugars and amino acids are usually in the ppb range or lower (Hodson 1977, Wright and Hobbie 1965). Fewer K_s values have been obtained for xenobiotic chemicals, but existing data do suggest that ppb values of K_s are not uncommon. For example, Button et al. (1981) recently obtained K_s values for acetylene, benzene, and ethylacetate degradation in seawater samples of 0.25 to 2.8 ppb. In the same study, V_{max} for degradation of toluene in seawater was reached at a substrate concentration less than or equal to 52.7 ppb, suggesting that K_s was significantly lower. Moreover, using estuarine water samples, Bartholomew and Pfaender (1983) have observed K_s values well below 10 ppb for several chemicals, including m-cresol and chlorobenzene. Baughman et al. (1980) state that K_s values typically range from 0.1 to 10 parts per million (ppm) in natural waters, but provide no references in support of that conclusion. Since concentrations of xenobiotics in natural waters are often in the low ppb range (Sheldon and Hites 1979), it is clear from the foregoing that [S] cannot be assumed to be much lower than K_s. Thus, it seems likely that equation (3) will in many cases not be approximated by equation (5). In those cases the simple second-order rate model represented by equations (5) through (8) is invalid.

The conclusion is not that this approach should be abandoned, but rather that more research is needed. Particularly needed is better knowledge of K_s values for biodegradation of organic chemicals by natural microbial populations. But more fundamentally, we need better knowledge of how microbial populations degrade organic chemicals at low, environmentally relevant concentrations. For example, evidence (Rubin et al. 1982) suggests that the bacteria responsible for biodegradation of organic chemicals at very low substrate concentrations (oligotrophic bacteria) may be different from those carrying out similar transformations at higher substrate levels, so that rate constants determined in studies using substrate concentrations of 0.1-10 ppm may not accurately relect degradation kinetics at lower concentrations.

PRACTICE

Regardless of theoretical difficulties, the simple second-order rate model may be useful as long as it "works." But inadequate validation remains a serious deficiency, and careless manipulation of environmental samples used in degradation studies can invalidate conclusions. With respect to validation, the model predicts that the rate of biodegradation of a substrate should be proportional to the microbial concentration, and that second-order rate constants should therefore be reproducible from site to site and sample to sample. Although a general relationship between population size and biodegradation rate is intuitively reasonable, few quantitative studies are available to support it. Paris et al (1981) established such a relationship for three chemicals, the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (2,4-DBE), malathion, and chlorpropham (CIPC). Site-to-site reproducibility of the second-order rate constants was excellent, with coefficients of variation for each chemical of less than 65% over all sites. Larson and Payne (1981) recently provided similar evidence for linear alkylbenzene sulfonates (LAS) degradation in samples of Ohio River water.

On the other hand, several studies have failed to demonstrate a significant relationship between biodegradation rate and microbial concentration, or have shown that other factors may be more important in determining biodegradation rates. In a study of biodegradation of several organic chemicals in fresh, marine, and estuarine waters, Bartholomew and Pfaender (1983) found that measurements of microbial numbers did not correlate very well with measurements of microbial activity, and in particular, with the maximum rate of utilization of the added substrate m-cresol. Nesbitt and Watson (1980a, b) studied the biodegradation of 2,4-dichlorophenoxyacetic acid in river water, and showed that degradation was primarily dependent upon microbial activity rather than the total number of microorganisms. They also demonstrated that microbial activity was related to the concentration of inorganic nutrients, the suspended sediment load, and the dissolved organic carbon content of the water.

Yordy and Alexander (1980) demonstrated that the rate of disappearance of N-nitrosodiethoanolamine in samples were collected. These authors suggested that the presence of competent microorganisms in water samples was more important than total population size. Boethling and Alexander (1979) observed apparent thresholds at ppb concentrations for degradation of certain organic chemicals in samples of stream water. These observations do not support a simple second-order rate model, since at low substrate concentrations biodegradadation rates should be directly proportional to the initial substrate concentration.

Spain et al. (1980) showed that adaptation of microbial populations by prior exposure to an organic chemical could have a marked effect on biodegradation rates observed in subsequent exposures to the chemical. Measurements of total microbial population size did not reflect the altered biodegradation rates. These and other studies show that environemtal factors may overshadow the effects of population size. This statement has important implications for prediction of biodegradation rates in natural waters. It says that a smiple second-order rate constant determined using a given water sample cannot be used to predict degradation rates at other sites, without consideration of factors other than total population size that may affect the rates.

It is significant that (1) the compounds studies by Paris et al. (1981) are known to be biodegraded initially by hydrolysis, catalyzed by enzymes that are probably constitutive, and (2) biodegradation was followed by measuring disappearance of parent compound. The choices of test compound and analytical method thus acted to maximize the probability that biodegradation rates would be proportional to total population size. It is reasonable to suppose that different results might be obtained if test compounds and conditions required degradation by less obvious routes. Would degradation rates always be proportional to total population size if the transformation of interest were carried out by few bacteria, via complicated, inducible pathways? Would strict proportionality be obtained if the concerted action of several different bacterial strains were required? These phenomena are very common. Couldn't prior exposure to the chemical of interest, or to chemicals having related structures, have greater impact on degradation rates than total proulation size?

Many of these problems could be avoided if there existed good methods for measuring not the total microbial population, but only that segment of the population active in carrying out the desired chemical transformation. Unfortunately, such methods are not available for most chemicals. This is an area in which innovative research is badly needed. In the absence of methods for measurement of transformer populations or activity, calculation of second-order rate constants must depend upon measurements of total population size. Examples of methods commonly used by microbiologists include plate counts, determination of total adenosine triphosphate (ATP), and direct counting by epifluorescence microscopy. Which method is used really doesn't matter, since they all suffer from the same drawbacks relative to their application to the simple second-order rate model.

The preceding discussion points to the need for much greater effort in the validation of the model. The most critical need is for testing of more compounds, representing a wide variety of chemical structures of varying complexity. The biodegradation of these chemicals should be studied using samples of natural waters that would be expected to present a variety of adaptation possibilities: water from streams receiving industrial effluents, pristine lakes, saltwater environments, etc. Furthermore, biodegradation should be determined by measuring a parameter such as CO_2 evolution in addition to disappearance of parent compound. The approach of Larson and Payne (1981) is encouraging in this regard, as these investigators used ring-labelled LAS and followed production of $^{14}CO_2$ from cleavage of the benzene ring. Ring cleavage requires the action of inducible enzymes. The choice of LAS may be criticized from the standpoint of model validation, however, since their use as detergents has no doubt led to the widespread development in nature of microbial populations competent to degrade them.

Another issue worthy of mention is related to the changes in microbial numbers and activity, population diversity, and nutrient concentrations, that usually occur during long-term incubation of environmental samples.

It has long been known, for example, that changes in population density of 100 fold or more can occur in samples stored for long or even short (less than 24 hours) periods of time, even when the samples are refrigerated (ZoBell 1946). Accordingly, certain precautions must be observed in any biodegradation study in which degradation in environmental samples is determined. Such changes are difficult or impossible to avoid in long-term biodegradation tests, but they can be minimized during the period from sample collection to the start of the test, and changes in microbial numbers over time, at least, can be easily followed.

It is therefore unfortunate that Paris et al. (1981) do not indicate how long or under what conditions wAter samples were held before they were received in the laboratory. Moreover, significant changes may have occurred during the 48-hour period between receipt of the samples and the start of biodegradation tests. Paris et al.(1981) state that bacterial numbers did not vary significantly over the course of their experiments. Such behavior could be expected if one or more days had elapsed between sample collection and the start of biodegradation experiments, because bacterial numbers in stored water samples typically increase rapidly, and then decrease very slowly over several days or longer (ZoBell 1946). Considering the fact that water samples were collected from 40 sites in 18 states, delays of one to several days would seem inevitable. If the microbial numbers thus determined do not reflect in situ values, second-order rate constants calculated from those numbers cannot have environmental significance for the sites from which the samples were collected.

SUMMARY AND CONCLUSIONS

The second-order model for prediction of biodegradation of organic chemicals in natural waters offers beguiling simplicity. One needs only to follow disappearance of parent compound and microbial concentration over time. The pseudo first-order rate constant is determined from a plot of ln chemical concentration vs. time, and the second-order rate constant is obtained from the pseudo first-order rate constant by dividing the latter by the microbial concentration. The second-order constant is presumably site and sample independent and can be used, given a measurement of mocrobial concentration for any site, to predict a half-life for the chemical of interest at that site. Structure-activity relationships can be established by correlation of the second-order biodegradation rate constants with other known physical/chemical or fate parameters, such as alkaline hydrolysis rate constants (Wolfe et al, 1980).

Numerous theoretical and practical problems intervene in the implementation of this model, however. Theoretical problems include the false assumption that the test chemical is growth limiting, the uncertain meaning of the yield coefficient Y in such cases, and probably most important, the potentially false assumption that natural chemical concentrations are much lower than K_s. Foremost among practical problems is that the model is as yet largely unvalidated. If one is willing to suspend reservations about their handling of water samples, Paris et al. (1981) have indeed demonstrated the site independence of second-order rate constants. But only three chemicals have been extensively tested, and the chemicals had structures for which some degree of site independence of the second-order rate constants should not be terribly surprising. Moreover, there exists a substantial body of evidence to suggest that, for many chemicals, various environmental factors can overshadow the effects of microbial numbers, thus precluding the establishment of significant correlations between total numbers and degradation rates.

The most urgent need is for more research to demonstrate the practical usefulness of the model. Many chemicals having a variety of structures should be studied, and proper attention should be paid to storage and handling of water samples. Concomitantly, microcosm studies should be initiated with the same chemicals, and the results from the two techniques compared. Until these experiments have been performed, it cannot be assumed that second-order rate constants are site independent, or even independent of sample for samples collected over time at a given site. If such independence cannot be shown, second-order rate constants offer no advantage over first-order constants.

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THE SECOND-ORDER APPROACH TO PREDICTIVE MODELING

John E. Rogers

ABSTRACT

To estimate the rate of degradation and thus the persistence of organic compounds in a wide range of environments will require an in-depth understanding of the microbiological transformation pathways (reaction sequences) of major compound classes and the effects of physical-chemical (environmental) parameters as well as microbiological population dynamics on the rates of these reactions. Although not complete, a wealth of information is available in the open literature describing the metabolism of major compound classes, especially in the area of aerobic degradation. Anaerobic metabolism has not been characterized to the same degree. The microbiological degradation and transformation rates of numerous organics have been determined in environmental samples. Unfortunately, much of this data base is sample specific and not transferable to other sites. Only in recent years have attempts been made to quantitate these rates so they can be used to estimate the persistence of specific organics in a range of environments. These studies have led to the development of a number of mathematical models to estimate persistence of organics.

DEVELOPMENT OF PREDICTIVE MODELS

The development of viable research and predictive models for the degradation of organic compounds in the environment requires the interactions of the following two research areas.

- The development of environmentally applicable mathematical representations of the microbiological processes involved in the transformation and ultimate degradation of organic compounds.
- The design and development of procedures to produce laboratory data compatible with the mathematical representations.

Previously these areas have, in many respects, developed independently. In the future, as microbiological degradation models become more sophisticated, it is essential that they be investigated in an interactive manner, each process in turn being directed by the other as new information and methodologies are developed. This will ensure the most rapid and complete development of useful microbiological models. This does not preclude theoretical studies. However, the development of models is generally of little practical value when no consideration is given to the availability of laboratory data to exercise the model or worse if no consideration is given to the development of laboratory techniques to provide the data. This is also true of degradation studies, where the usefulness of the data, as input for available or developing computer models, has not been considered. Continued efforts to integrate these two areas will lead to better microbiological models and a better understanding of microbiological degradation in the environment.

An important first step in this direction has been to use a pseudo second-order rate equation to describe the microbiological transformation of organics in fresh and marine waters. There are two constraints, however, to the use of this equation: the substrate concentration must be less than saturating (assuming that microorganisms can be considered catalysts) and it must not support growth of the microorganisms. The utility of this equation has been demonstrated over a large number of waters for several compounds which include a number of phenol derivatives (Paris et al. 1982), malathion, and chloropropham (Paris et al. 1981) as well as butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (Paris et al. 1981; Rogers et al. 1983). The pseudo second-order rate constants were reproducible within ~65% for the different waters examined within a given laboratory. This reproducibility offered significant encouragement for the prediction of the environmental fate in water of organics that met the constraints listed above.

Early in these studies it was observed that certain compounds were transformed only after a variable and concentration dependent lag or adaptation period. In the laboratory pseudo second-order rate constants are determined by dividing the measured pseudo first-order rate constant by the bacterial concentration. The pseudo first-order rate constant is obtained from the slope of a log (residual organic) vs. time plot. When adaptation to a compound occurs, the pseudo first-order rate constant is determined from the linear portion of the log plot. It was immediately apparent from these studies that the use of second-order rates to model the degradation of compounds which first require adaptation is only a partial model. A complete model would include a mathematical expression for adaptation and the transformation rate. For situations where growth is occurring during adaptation, the curve can be represented by the Monod growth equation for example. In situations where growth does not occur but the organic substrate concentration is saturating, the resulting data can be represented by the Michaelis-Menten equation for enzyme kinetics. We have observed that for some compounds the adaptation period is far greater than the time during which degradation occurs. In this situation one can simply state that the compound resides in the environment at a specific concentration for a specific period of time.

UTILITY OF SECOND-ORDER MODELS

The potential utility of the second-order models rests on the ability of laboratory studies to determine an average pseudo second-order rate constant, using a number of different waters with different microbial populations. The average rate constant can then be used to predict degradation rates at other sites if the microbial population and organic substrate concentrations are known. This combined methodology can also be used when the degradation rate is described by alternative mathematical expressions. For example, if the Michaelis-Menten equation (1) were used, one would simply determine the average values for K_m, V_{max} and the bacterial population of a number of waters and then use these average values to estimate degradation rates in other waters where the substrate concentration and bacterial populations are known. The bacterial population in this case is used to set the value of V_{max} from an average specific activity term (sp. act. = V_{max}/bacterial population) determined in the initial evaluation studies. Similar examples can be derived from other mathematical expressions for the degradation rate.

A continued research effort in this area, which I will term the "second-order approach to predictive modeling," should address the areas of adaptation kinetics, reaction kinetics, and quantitation of biomass. Biomass has been included because it becomes important when extrapolating from basic research models to widely applicable predictive models which are normally constructed to use readily available or easily attainable data. For example, the potential for the degradation of organic compounds cannot be quantitated by using a most probable number technique (MPN) which utilizes 14-C-labeled substrates. Using this technique, one obtains a compound specific biomass measure. 14-C-MPN techniques can be expensive. However, this technique can be shown to be proportional to the total bacterial colony-forming units obtained by standard water quality methods; for example, the initially costly 14-C-MPN data base can be expanded cost effectively to a wider range of environments. This would make the best use of 14-C-MPN which is a good research tool and standard plate count methods which is a widely used nonspecific enumeration technique.

SUMMARY

A research effort should include at least the following set of tasks and subtasks.

Task 1. Identify adaptation phenomena for different compound classes at different concentrations.

Subtask 1. Classify compound classes as to 1, 2, and 3 below.

1. No adaptation.
2. Adaptation is approximately equal to half the time required to degrade the compound.
3. Adaptations are markedly longer than time required for degradation.

Subtask 2. Define the kinetics of the adaptation process (growth, adjustment to compound, induction, etc.)

Subtask 3. Define computer inputs and mathematical expression for different adaptation processes (e.g., Monod kinetics which require input of binding constants and specific growth rates).

Task 2. Identification of specific transformation kinetic processes.

Subtask 1. Characterize compound classes as to pseudo first-order, pseudo second-order, and Michaelis-Menten kinetics.

Subtask 2. Define computer inputs and mathematical expressions for different kinetic processes.

Task 3. Develop site independent biomass measures.

Subtask 1. Develop compound specific biomass measures (e.g., MPN).

Subtask 2. Develop general biomass measures which relate to specific measures (e.g., cfu).

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USE OF FIRST- AND SECOND-ORDER MICROBIAL TRANSFORMATION RATE COEFFICIENTS FOR PREDICTIVE MODELS

David L. Lewis

ABSTRACT

In our research at the Athens-ERL, we have assumed that microbial transformations of xenobiotic chemicals are a result of enzymatic reactions, thus following Michaelis-Menten kinetics. Based on Michaelis-Menten kinetics, we have used first- and second-order transformation rate coefficients for predicting transformation rates by suspended, attached, and sediment-associated microbiota. Our second-order transformation rate coefficients have been determined using either total plate counts or transformer enumerations for suspended organisms (1), including blended aufwuchs (2) and ratios of surface area to container volume for surfaces, including sediments, covered with attached organisms (3,4,5).

APPLICATION OF TRANSFORMATION RATE COEFFICIENTS

The application of transformation rate coefficients to predicting transformation rates in field situations utilizes numerous assumptions relating to (i) the applicability of the Michaelis-Menten equation and its derivative equations, (ii) the use of correlative parameters such as plate counts, direct counts, and colonized surface areas for determining second-order transformation rate coefficients, and (iii) the effects of various environmental parameters on transformation rates. The strengths and weaknesses of these assumptions have been discussed in a recent manuscript on the application of single- and multiphasic-kinetics to predictive modeling for aquatic ecosystems (6). An analysis of these assumptions has led us to conclude that in addition to further research on kinetics, other areas need to be investigated to improve our predictive capabilities. These areas include (i) suppression of microbial transformations of xenobiotic chemicals by diauxie, biologically produced inhibitors, and other xenobiotic chemicals; (ii) adaptation; (iii) mass-transport effects; and (iv) multiphasic kinetics.

FUTURE RESEARCH NEEDS

In general we feel that transformation rate coefficients based on Michaelis-Menten kinetics are most applicable for predicting transformation rates of xenobiotic chemicals in low environmental concentrations. A K_m value as low as 10^{-7} $\underline{M}$ was observed for transformation of diethyl phthalate (Figure 1). Therefore, the maximum concentration of xenobiotic chemicals for which microbial transformation rates can be predicted without the confounding effects of saturating some or all of the enzyme systems may be as low as 10^{-7} $\underline{M}$. Transformation rates of higher xenobiotic chemical concentrations may also be confounded by multiphasic kinetics, toxic effects of the xenobiotic chemical, and adaptation. Figure 2 shows the general divisions of confounding effects and applicable transformation rate coefficients for various xenobiotic chemical concentration ranges.

We believe that transformation rate coefficients may be satisfactorily estimated using SAR (7,8,9), or by using relative transformation rate coefficients compared to coefficients determined for microbial transformations of reference chemicals (6).

There have been many frustrations associated with taking simple approaches to predicting microbial transformation rates. These frustrations have tended to compel us toward increasing the sophistication of our approaches. However, we believe that our methodology must incorporate analytical methods commonly available to industries and governmental agencies, thereby preserving practicality. We hope to demonstrate that even diverse and complex environments operate within sufficiently narrow bounds and without an inordinate number of essential principles so that good estimates of transformation rates can be made using relatively simple models.

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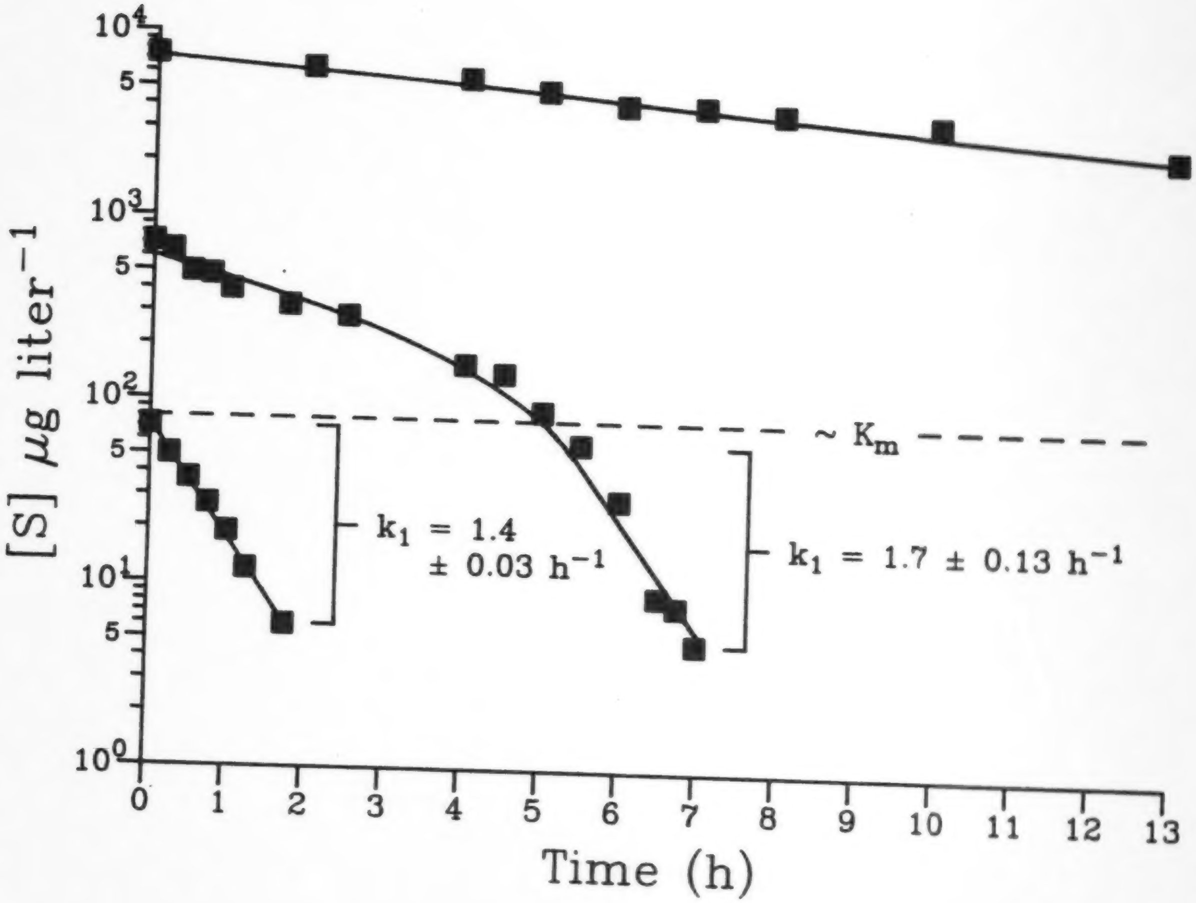
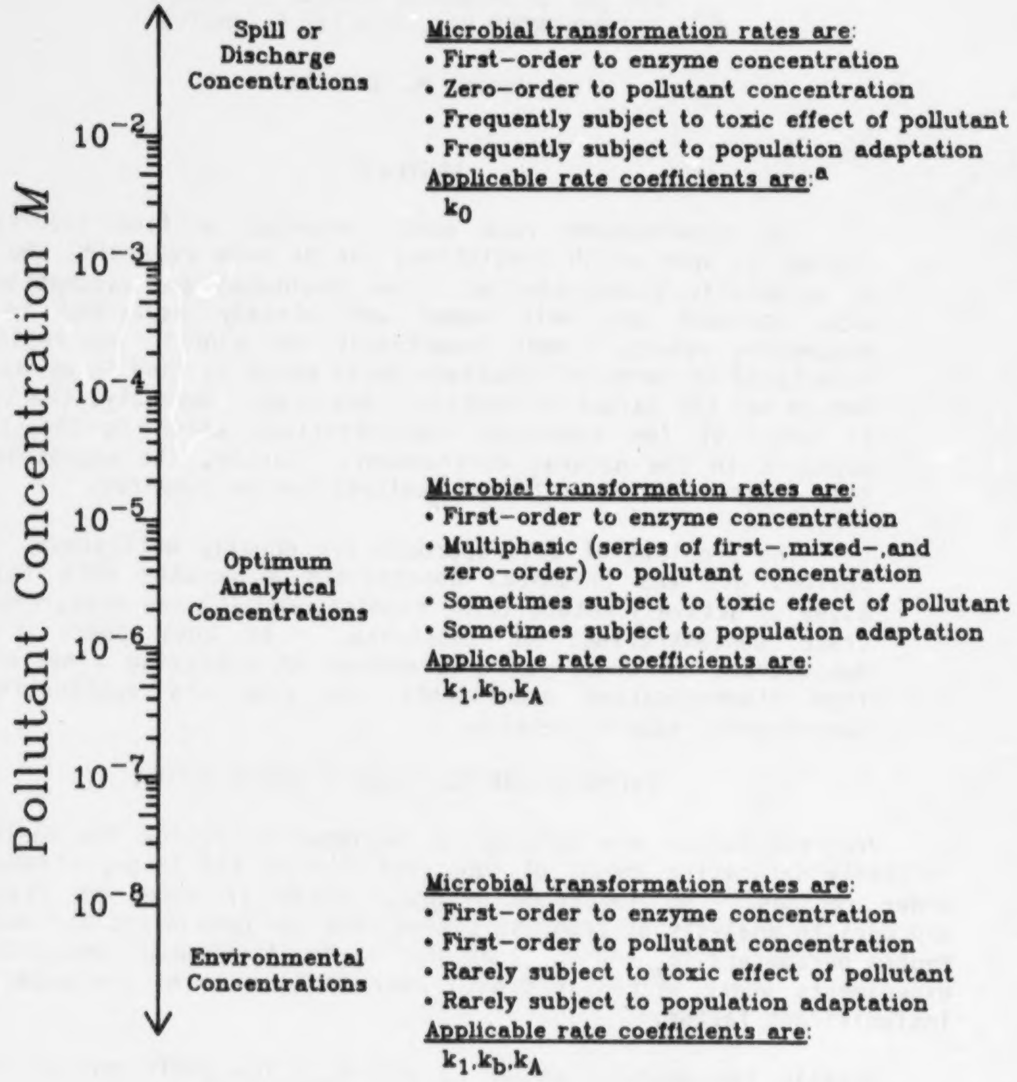


Figure 1. Saturation of Brevibacterium sp. with diethyl phthalate (DEP). K_m was approximately 80 ug liter^{-1} (4 X 10^{-7}M).


Pollutant Concentration M
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
Spill or Discharge Concentrations
Optimum Analytical Concentrations
Environmental Concentrations

Microbial transformation rates are:

- First–order to enzyme concentration
- Zero–order to pollutant concentration
- Frequently subject to toxic effect of pollutant
- Frequently subject to population adaptation

Applicable rate coefficients are:[a]

k_0

Microbial transformation rates are:

- First–order to enzyme concentration
- Multiphasic (series of first–,mixed–,and zero–order) to pollutant concentration
- Sometimes subject to toxic effect of pollutant
- Sometimes subject to population adaptation

Applicable rate coefficients are:

k_1, k_b, k_A

Microbial transformation rates are:

- First–order to enzyme concentration
- First–order to pollutant concentration
- Rarely subject to toxic effect of pollutant
- Rarely subject to population adaptation

Applicable rate coefficients are:

k_1, k_b, k_A

---

[a] Transformation rate coefficients are based on rates of loss of parent compound and include k_0, mg liter^{-1} h^{-1} per biomass concentration or per ratio of periphyton–colonized surface area to volume; k_1, h^{-1}; k_b, liters cell^{-1} h^{-1}; and k_A, liters m^{-2} h^{-1}.

Figure 2. Summary of microbial transformation rate information based on Michaelis-Menten kinetics, experimental data, and various assumptions used in the Exposure Analysis Modeling System (EXAMS).

THE USE OF PROGRESS CURVES TO OBTAIN SECOND-ORDER RATE KINETIC ESTIMATES

Joseph M. Suflita

ABSTRACT

The second-order rate model provides a firm theoretical foundation upon which predictions can be made regarding the rate of xenobiotic biodegradation. The advantages and assumptions of this approach are well known and already explained in the preceeding papers. Most importantly the kinetic expression is formulated in terms of substrate decay which is (and in my opinion should be) the parameter routinely measured. Secondly, the theory is valid at low substrate concentrations which is the likely scenario in the natural environment. Lastly, the expression is site independent so different habitats can be compared.

The problems of this approach are equally well known. Most obvious are the practical constraints associated with (a) the assay of actively metabolizing biomass, and (b) the measurement of trace concentrations of substrate. In this paper I will demonstrate an under-utilized method of analyzing kinetic data from biodegradation experiments and show its application to second-order rate expression.

INTRODUCTION TO PROGRESS CURVE THEORY

Progress curves are experiments designed to follow the depletion of an initially saturating amount of substrate through the zero-, mixed- and first-order regions. An idealized progress curve is shown in Figure 1. By appropriate analysis of progress curves, one can obtain the various Michaelis-Menten parameters (K_m and V_{max}) as well as the first-order decay constants for experiments where microbial growth over the course of the experiment is an insignificant factor.

Usually the calculation of K_m and V_{max} are performed by using one of several linearizing transformations of the Michaelis-Menten equation such as the Lineweaver-Burk double reciprocal plot. This approach requires several substrate-velocity data pairs and conclusions are usually drawn from 5 or 6 data points at best. It is becoming well known that the parameter estimates using these techniques can be notoriously inaccurate.

However, such difficulty is partially circumvented through the use and analysis of progress curves. Because the Michaelis-Menten model is a differential velocity equation, its integrated form is valid over the entire course of the reaction. The apparent K_m and V_{max} parameters can be determined by measuring the loss of substrate (or production of product) several times during the course of the experiment incorporating as many data points as desired. The information is then plotted using the integrated Michaelis-Menten equation (Figure 2). From such a plot, or one of several other linearizations of the Michaelis-Menten equation, one can easily derive the Michaelis parameters (K_m and V_{max}) as well as the first-order decay rate (V_{max}/K_m).

APPLICATION OF PROGRESS CURVES TO EXPERIMENTAL DATA

Figure 3 shows a progress curve for the anaerobic degradation of 4-amino-3,5-dichlorobenzote by sediment microorganisms. Using the same data set, the information was plotted using several linearizations of the integrated Michaelis-Menten equation. The various equations and the associated regression coefficients are shown in Figure 3. I purposely chose an equation which did not give the highest R^2, estimated the kinetic parameters K_m and V_{max}, and then used a computer to simulate a progress curve based on those kinetic estimates. The solid line is computer simulated, and the data are superimposed on it. As can be seen, the fit is fairly good, giving a K_m estimate of 30μm and a V_{max} estimate of 1.5 μmoles $l^{-1}h^{-1}$.

Let's examine another progress curve, Figure 4a, this time following the anaerobic dehalogenation of 3-chlorobenzote by an enriched methanogenic consortium. On this arithmetic plot we can get a fairly good straight line through most of the data, suggesting that it is zero-order. If we are truly in the proper substrate range, we should get the curve shown in Figure 4b, a semi-log plot. Note that if a regression line was drawn through the initial data points, we would still get a fairly good straight line with an R^2 of about 0.98. In this particular case it covers at least one order of magnitude before the curve bends off going to the true first-order region. The same is true for the degradation of 3,5-dichlorobenzote (Figure 5). The same basic pattern is seen: again quite a long region of linearity before the curve gradually enters the first-order region.

We can analyze this progress curve data and generate the Michaelis-Menten parameters and simulate progress curves based on our kinetic estimates. As can be seen in Figure 6, we can estimate K_m's of about 67 and 47 μM for the mono and dichlorosubstrates respectively and V_{max} values of about 24 and 8. The data in this figure are superimposed on the computer simulated solid line based on the kinetic estimates. As can be seen, the fit is fairly good in both cases. The important point is that the kinetic parameters can be estimated from a single experiment.

Several questions arise. Can this approach be extrapolated to more complex systems other than enrichments? How does it all relate to second-order rate theory? Knowing K_m and V_{max} we can easily calculate a first-order decay constant which is simply V_{max}/K_m. According to second-order rate theory, we need to normalize the first-order rate by the biomass concentration. The open question is what do we use as a measure of the biomass which is actively involved in substrate metabolism?

In the saturating or zero-order region of substrate decay, the rate of degradation is independent of substrate concentration and dependent on the amount of catalyst. Therefore, the zero-order rate of substrate decay should be an accurate reflection of the quantity of active catalyst in the system. The zero-order rate is simply the V_{max} term. We then need experimental verification of this model. Ideally, we should be able to experimentally vary biomass, run progress curves at initially saturating substrate concentrations, and normalize the observed first-order decay rates by the zero-order decay rates.

This can be seen in an experiment done with anoxic sediment slurries. The amount of biomass was varied by dilution with filter sterilized anaerobic lake water. Progress curves were performed on these samples with 4-amino-3,5-dichlorobenzoate as substrate and kinetic estimates were obtained using the integrated Michaelis-Menten equation. As can be seen in Figure 7, dilution had very little effect on the biochemistry of the reaction; the apparent K_m was estimated to be about 30 μM in each case. However, dilution did have an affect on the amount of active biomass and therefore the V_{max} of the reaction. It was approximately halved in the diluted sediment slurry. If the first-order decay rate is then normalized by the maximum velocity, then essentially the same second-order proportionality constant is obtained. This evidence supports the contention that the rate of biodegradation is proportional to both substrate concentration and biomass and that V_{max} estimates are a reasonable measure of the concentration of active catalytic units. It therefore does not matter if the active catalyst is an exoenzyme, individual cells, or a consortium of organisms.

Table 1 illustrates that there is fairly good agreement between the expected and measured rates of 3-chlorobenzoate metabolism when the biomass content of sediment slurries was varied by dilution. Thus, a decrease in biomass resulted in a corresponding drop in the zero-order rate of decay.

FUTURE RESEARCH NEEDS

In conclusion, it can be seen that progress curves can be used to obtain parameter estimation of the salient kinetic constants including the second-order rate constant. In addition, it is believed that the V_{max} of a reaction provides a reasonable estimate of the concentration of active catalyst.

The advantages of using progress curves are:

a. Kinetic estimations are derived from a single experiment.
b. With accurate K_2 values established with a prior research effort, any lab can measure V_{max} and make predictions at any substrate concentration; no need to go to low substrate concentrations.
c. Expressions can be amended mathematically to account for other factors like partitioning, heterogeniety, etc. (see below).
d. Conservative rate estimations can be made.

The disadvantages of progress curve analysis are:

a. It may be impossible to get saturation for some substrates.
b. There is no accounting for lag or acclimation.
c. Math is sensitive to "renegade" points.

It is also assumed in the use of progress curves that a decrease in reaction velocity is due only to decreasing saturation of the catalyst. In addition, Michaelis-Menten kinetics only describe substrate consumption when this process is either (a) unlinked to growth, or (b) when the amount of growth occurring is less than that which gives sigmoidal substrate depletion curves.

It is very important in performing progress analysis to:

a. Choose saturating initial substrate concentrations (i.e., 2-4 K_m) and follow degradation to first-order region ($0.1K_m$).
b. Take at least 10 data points covering the range in #1.
c. Use the linearized form of the integrated Michaelis-Menten equation or nonlinear least-squares analysis of data to get parameter estimations.
d. Simulate a progress curve based on the derived estimates of K_m and V_{max} to test reliability of parameter estimates.

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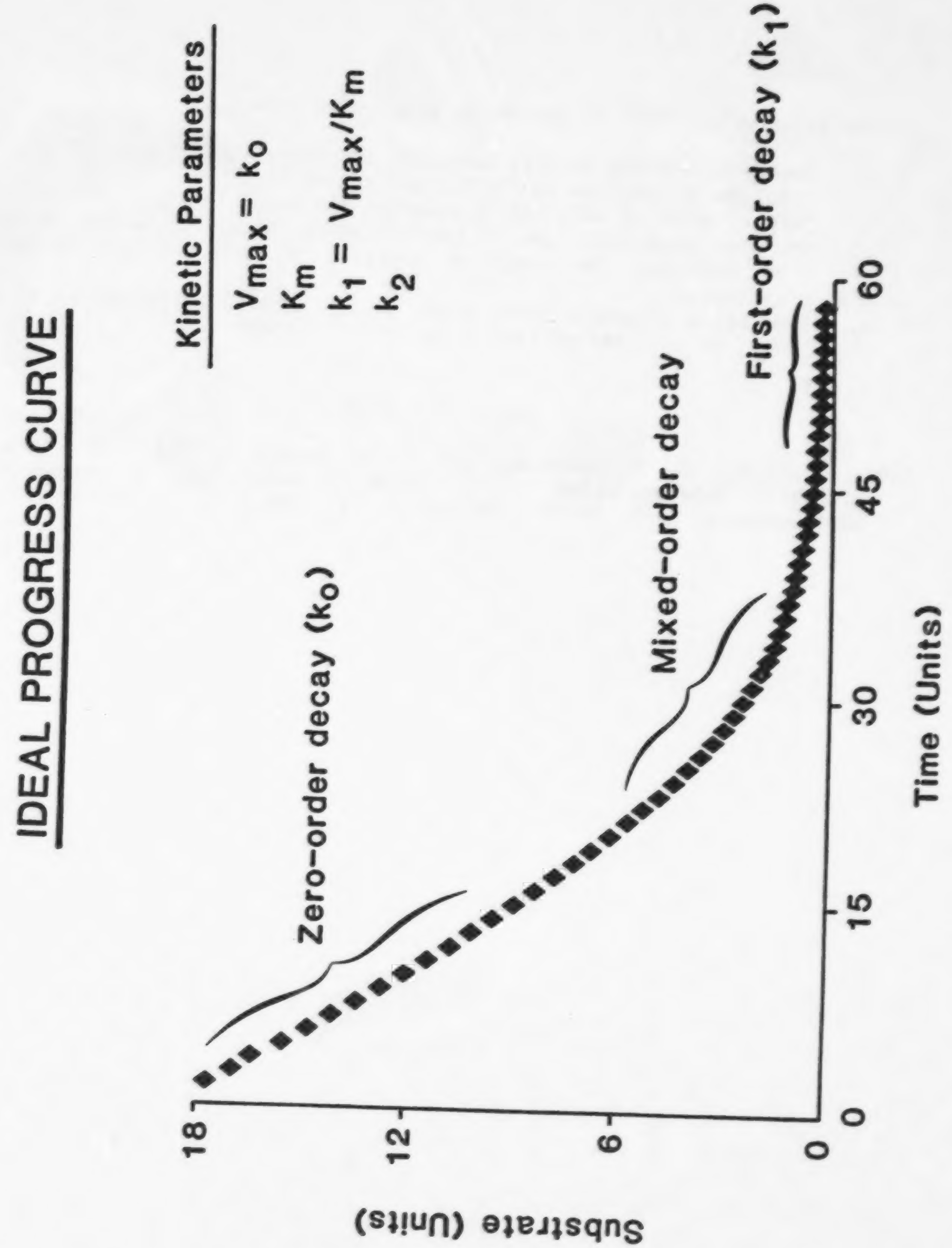


Figure 1. Idealized Progress Curve.

IDEALIZED PLOT OF INTEGRATED MICHAELIS–MENTEN EQUATION

$$(\ln S_o/S)/t = (-1/K_m)(S_o - S/t) + V_{max}/K_m$$

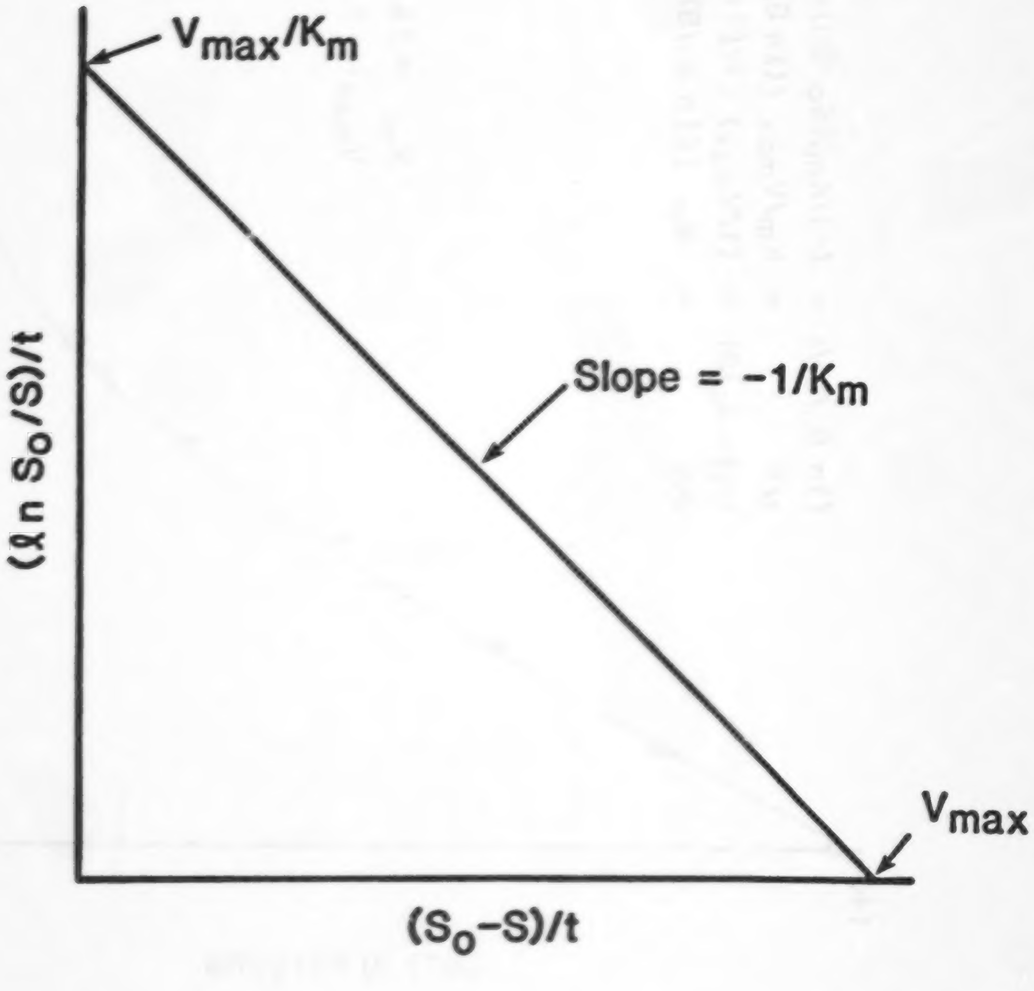


Figure 2. Idealized plot of integrated Michaelis-Menton equation.

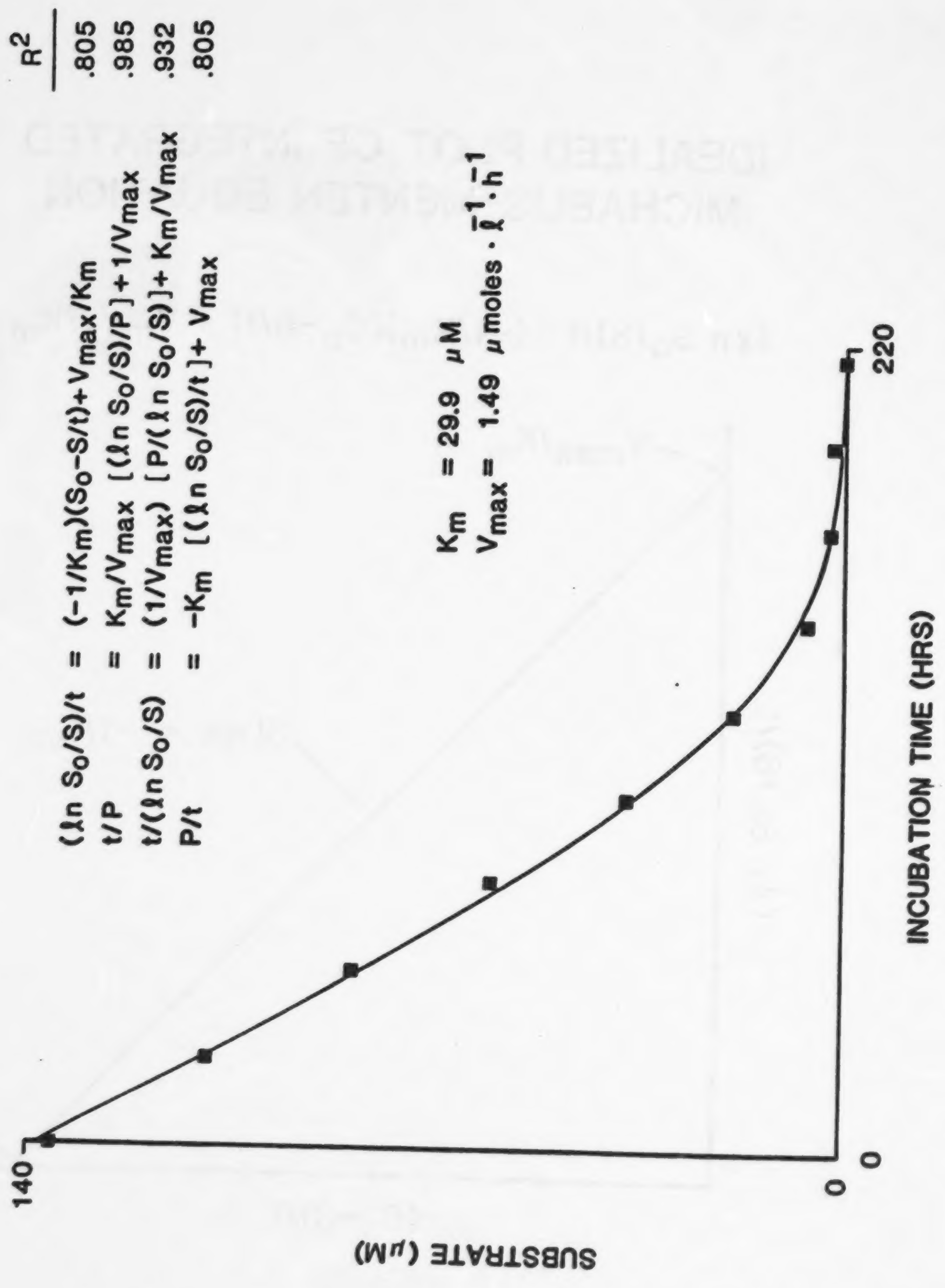


Figure 3. Progress curve for the anaerobic degradation of 4-amino-3,5-dichlorobenzoate by sediment microorganisms.

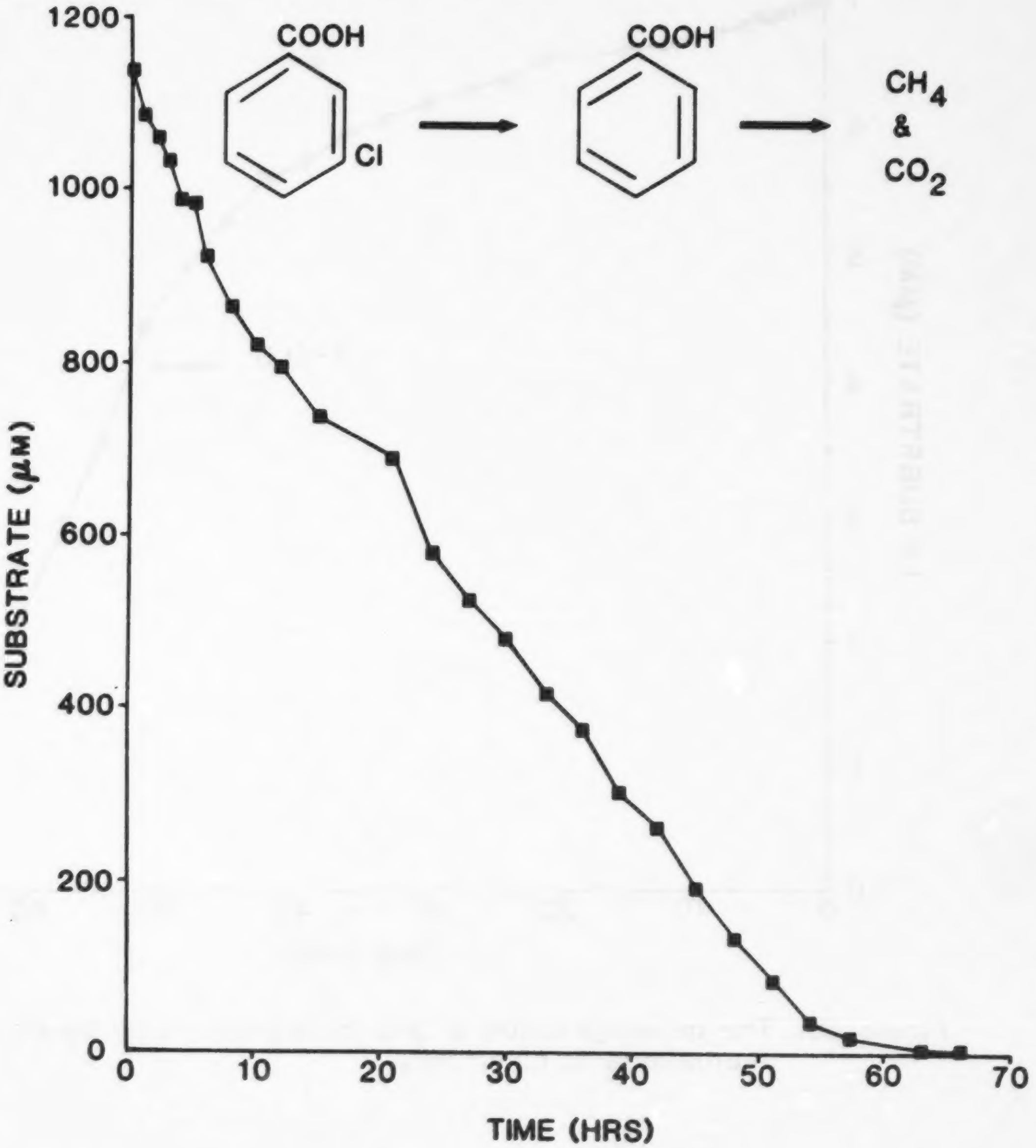


Figure 4a. Anaerobic dehalogenation of 3-chlorobenzoate by an enriched methanogenic consortium.

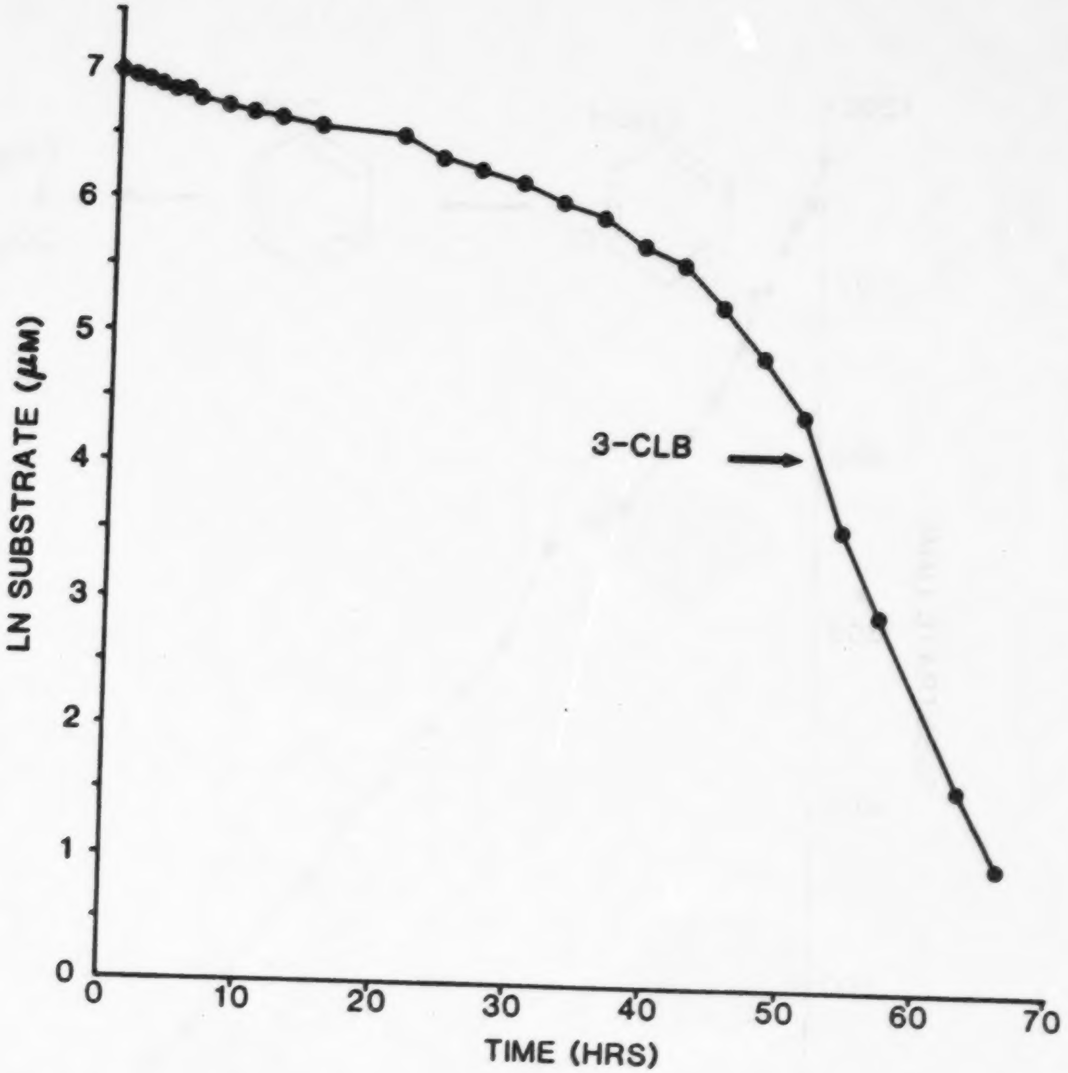


Figure 4b. The dehalogenation of 3-chlorobensoic acid by an enriched methanogenic community.

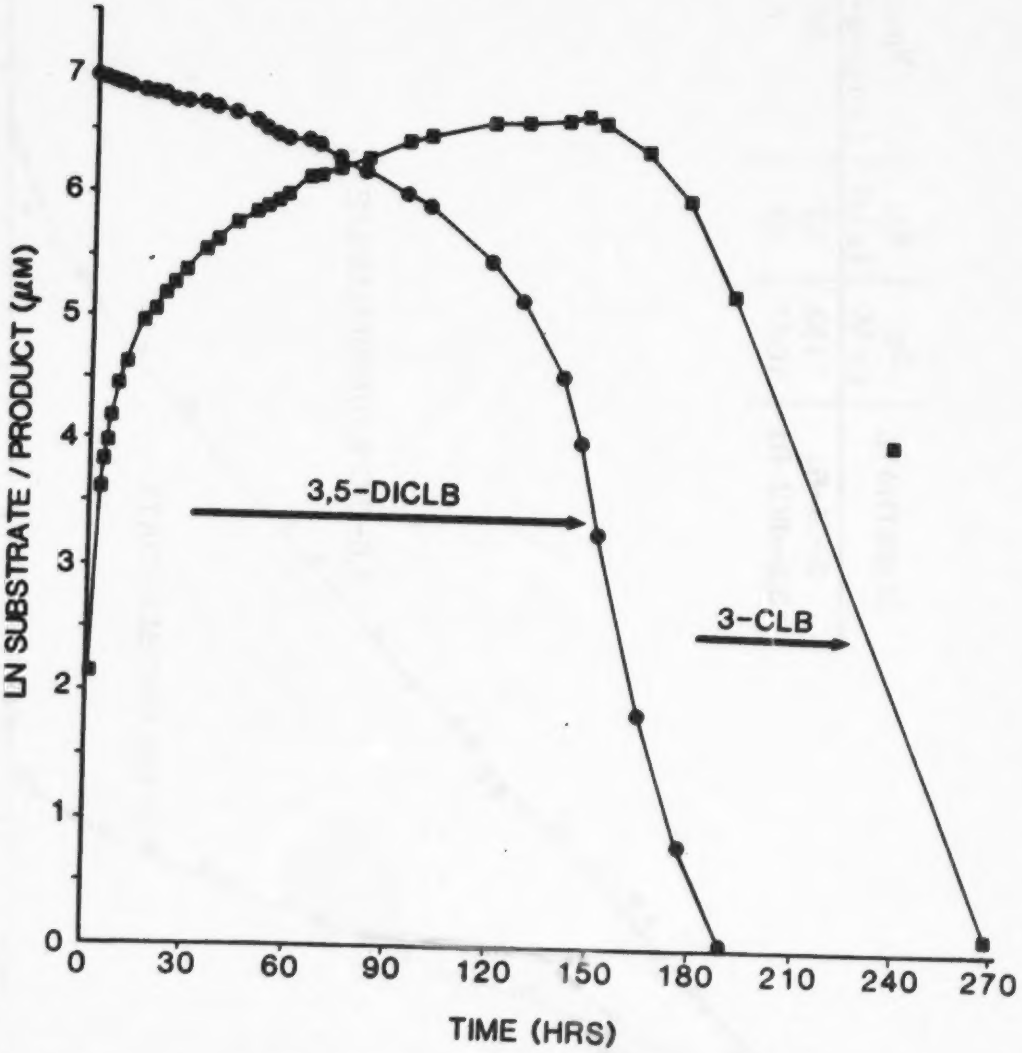
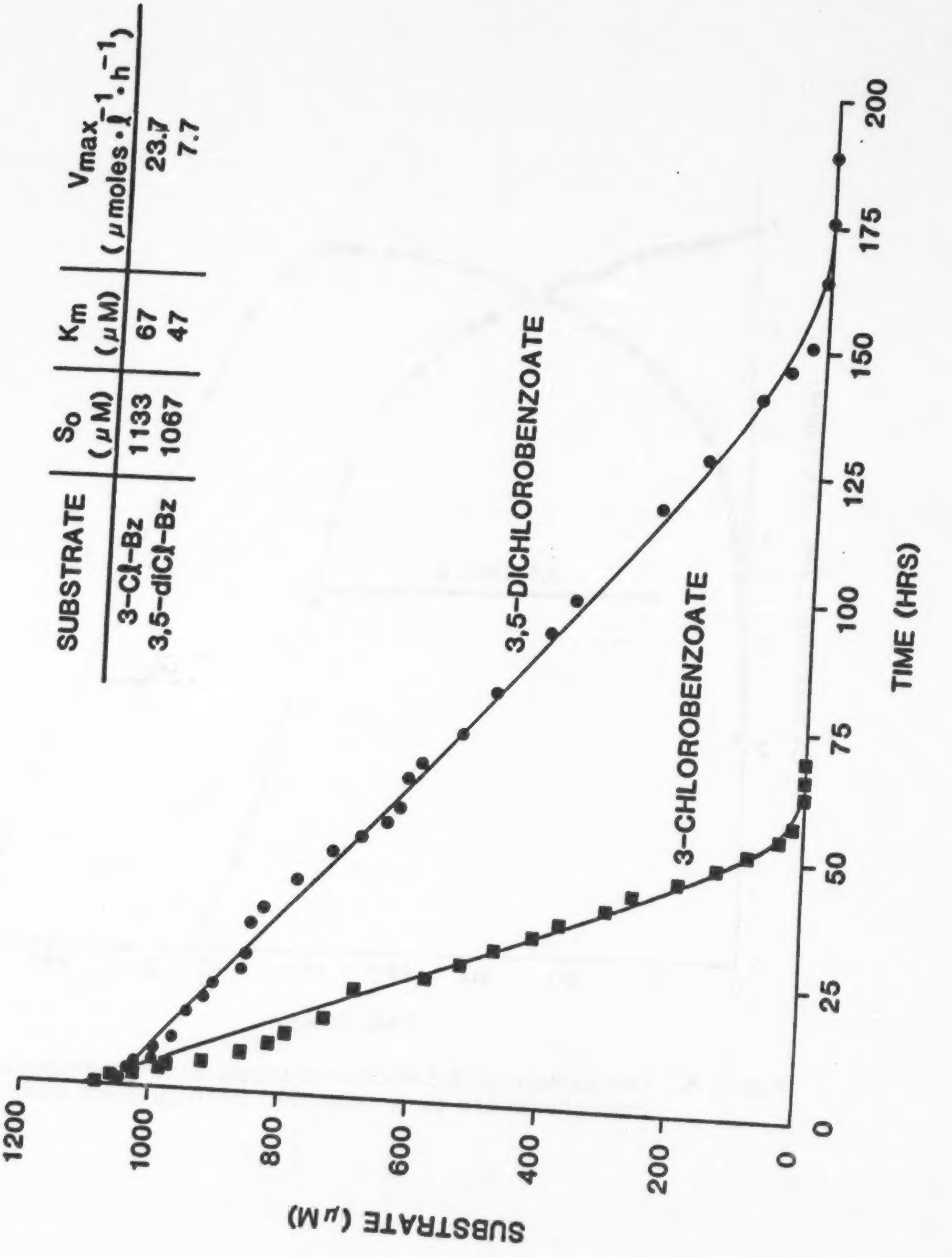


Figure 5. The pattern of 3,5-dichlorobenzoic acid dehalogenation to 3-chlorobenzoate by an enriched methanogenic community.



SUBSTRATE	S_o (μM)	K_m (μM)	V_{max} (μmoles $\cdot$ l^{-1} $\cdot$ h^{-1})
3-Cl-Bz	1133	67	23.7
3,5-diCl-Bz	1067	47	7.7

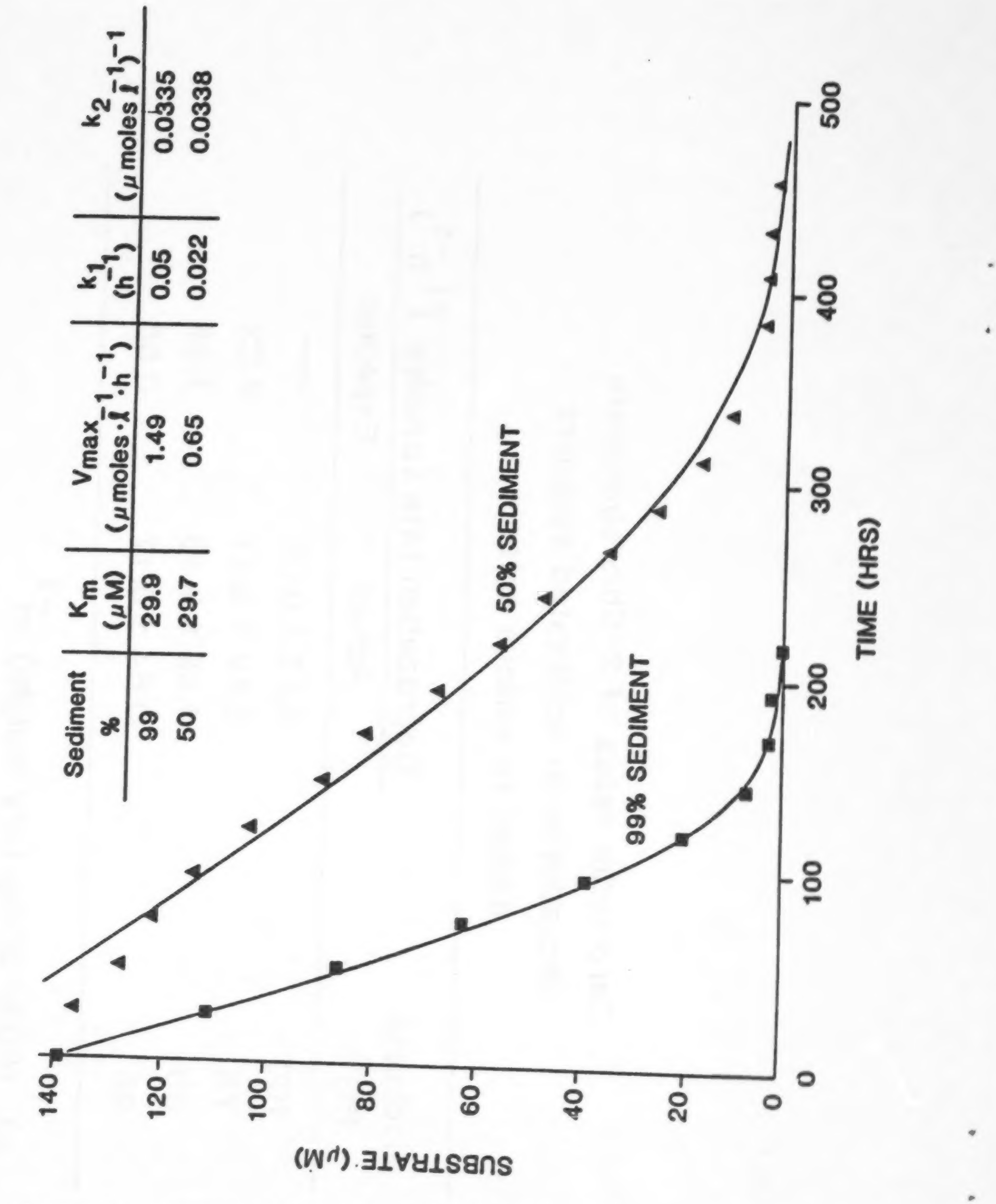
Figure 6. Simulated progress curves on mono and dichlorosubstrates determined by the kinetic parameters.

TABLE 1.

Zero-order rates of 3-Chlorobenzoate degradation in acclimated sediment diluted to various extents

Sediment (%)[a]	Degradation rate (μmoles l^{-1} h^{-1})	
	Actual	Expected
100	2.71 ± 0.08	—
75	2.32 ± 0.11	2.03
50	1.45 ± 0.13	1.35
25	0.64 ± 0.17	0.68

a) 100% = 50mg (dry weight) ml^{-1}



Sediment %	K_m (μM)	V_{max} (μmoles$\cdot l^{-1}\cdot h^{-1}$)	k_1 (h^{-1})	k_2 (μmoles l^{-1})$^{-1}$
99	29.9	1.49	0.05	0.0335
50	29.7	0.65	0.022	0.0338

Figure 7. Progress curves for the 4-amino-3,5-dichlorobenzoate by using the integrated Michaelis-Menten equation.

PANEL REPORT

The term "second order," previously used to describe a particular approach to site-independent biodegradation kinetics, was felt to be misleading and should be changed to "site-independent biodegration kinetics." The term "second order," developed from initial studies by utilizing a second-order rate equation derived from Monad kinetics, is now considered to be limited to the number of compounds and sites for which it is applicable. A site-independent mathematical approach should examine a number of potential kinetics expressions, letting the data dictate which expression is to be used by testing the fit of these equations by nonlinear regression. In a practical sense, the approach would use a series of equations for which the appropriate constants (K_m, V_{max}, K_2) have been developed in preliminary laboratory studies, and which given some simple measure (active biomass, substrate concentration, soil moisture, etc.) for the specific site, could then be applied to a wide variety of environments.

Research needs may be divided into two major areas:

1. The range of chemical structures and habitats studied to date has not been adequate to define the usefulness of this approach. This is particularly true with respect to structure, since most of the compounds for which we have data are degraded initially by hydrolytic enzymes. We should now focus on compounds such as chlorinated hydrocarbons and heterocyclics that are degraded by other routes. Of general importance is that the compounds selected do not closely resemble common naturally occurring compounds or other compounds expected to be ubiquitous in aquatic environments. It is also important that second-order rate constants be obtained for a wide variety of aquatic, terrestrial, and subsurface environments. The rate constants should then be compared not only within but also across environmental types to determine the degree of site independence.

2. An important gap in our knowledge concerns the length of the lag or acclimination period before degradation is observed. To our knowledge, there is no firm theoretical or mathematical foundation that can be used to predict when a particular compound may start to degrade. We suggest that research be directed to understand the reasons for acclimation periods and develop the appropriate descriptive mathematical equations. It would be extremely useful if the site independent microbial kinetic approach could be used to predict not only biotransformation rates, but also acclimation periods. As an initial approach, it might be worthwhile to try to correlate acclimation periods with some measure(s) of biomass.

As general guidance we also offer the following suggestions:

1. The methods used for measuring catalyst concentrations represent an area of special concern in any study designed to evaluate the usefulness of the second-order approach. We suggest that simple measures of total biomass such as plate counts or ATP levels be tried first. It should be recognized, however, that measures of total biomass may not be appropriate for many chemicals. Thus,

measures of specific degraders or, even better, of degrader activity are preferred. Possible parameters include V_{max} values, specific degrader MPN's, and microbial numbers as determined by microautoradiography. There may be other parameters and methods that give even better results, however, and these should also be explored.

2. Test chemical concentrations should range from very low to much higher levels. Whereas very low concentrations simulate conditions in many aquatic environments, high concentrations may be more relevant near point source discharges, in spill situations, or in areas receiving leachate from hazardous waste dumpsites.

3. The analytical regimen used to measure biodegradation should be versatile, so that (a) the complete mineralization of the substrate can be detected and quantified should that eventuality occur, and (b) parent compound depletion and product formation can be measured if complete mineralilzation is not the ultimate fate of the parent compound. A mass balance should be established in all studies.

4. To estimate variability, one needs an adequate number of replicates for each environment. Usually three to six replicates are considered adequate.

Panel Members: Martin Alexander, Chairman
Bob Hodson, University of Georgia
Dennis Focht, University of California
Dennis Laskowski, Dow Chemical
Bill Walker, Gulf Coast Research Laboratory
Herb Fredrickson, Georgia State University
Tom Federle, University of Alabama in Birmingham

FACTORS CONTROLLING BIODEGRADATION RATES IN MICROBIAL COMMUNITIES

Objective: To examine the physical and biological factors which control the rates of biodegradation and discuss how these rate-controlling factors can be incorporated into kinetic expressions of biodegradation. To develop a strategy for future research.

Biodegradation studies frequently produce chemical disappearance curves which reflect rate limitations due to such factors as changes in population size or composition, adaptation, competition for substrates, cometabolism, sorption to surfaces, and inorganic nutrient concentrations. What evidence suggests that these factors will be important in predicting biodegradation rates in the field? Can the effect of these factors be deciphered from the shape of the decay curve? What experimental techniques will provide more quantitative information on these rate-controlling factors? Can we assume that the size and activity of a degrader population within a microbial community is constant from site to site? Is it important to develop kinetic expressions which will accommodate different rate-controlling factors? What research is required to correctly model these special aspects of biodegradation?

UPTAKE OF DISSOLVED ORGANIC COMPOUNDS
BY AQUATIC MICROHETEROTROPHIC POPULATIONS:
MULTIPHASIC AND SIMPLE MICHAELIS-MENTEN KINETIC PATTERNS

Robert E. Hodson

ABSTRACT

Concentrations of individual dissolved organic compounds in natural waters are very low, in the range of picomolar to, at most, micromolar. The low ambient concentrations are due to efficient uptake of the organic substrates by microorganisms possessing specific, high affinity transport systems. When these systems are examined individually, most are found to possess finite V_{max} and K_t values and, thus, to exhibit simple Michaelis-Menten uptake kinetics. Since the mid-1960's, when radiotracer studies of the uptake of dissolved organic compounds from natural waters were first carried out, and until recently, it has been assumed that natural mixed-species assemblages of microorganisms also exhibited simple Michaelis-Menten uptake kinetics. This assumption formed the basis for models used to predict the fate of both natural and pollutionally-derived organic compounds in freshwater and marine environments. Recent evidence, however, indicates that natural assemblages of aquatic microheterotrophs possess a high degree of "kinetic diversity" when presented with dissolved organic compounds. Simple hyperbolic kinetic patterns, indicative of the entire population's having a single K_t and V_{max}, are observed less frequently than are complex, multiphasic kinetic patterns indicative of the presence of a wide variety of individual transport systems within the population.

INTERPRETATION OF KINETIC PATTERNS

Wright and Hobbie (1966) were among the first to study intensively the relationships between the concentrations of dissolved organic substrates in natural waters and the rates at which these substrates were taken up by the natural aquatic microbial populations. They employed a linearization plot derived from studies of enzyme-substrate interactions to determine graphically kinetic parameters for the uptake of organic compounds. In this procedure the value t/f, the incubation time (t) divided by the fraction (f) of labeled substrate taken up during the incubation, is plotted versus (A), the concentration of added substrate. The experimental procedures used initially involved determining t/f over a relatively narrow range (e.g., between one and ten micromolar) of substrate concentrations. The resulting data often fit a straight line fairly well, a fact that was interpreted as indicating that uptake was via a single transport system. With the Wright-Hobbie representation of the uptake data, the slope of the line of best fit is taken to be the inverse of the V_{max}; the Y-intercept is the _in situ_ turnover time for the substrate, and the X-intercept is equivalent _to the_ sum (K_t + Sn) where Sn is the _in situ_ substrate concentration.

After this approach became widely used, the data were nearly always treated as though the relationship between t/f and (A) was theoretically linear no matter how well the experimental data fit a straight line. It became generally assumed that the microbial population in a particular body of water possessed a single K_t and V_{max} for uptake of a given compound. Concentrations of microbially utilizable compounds are exceedingly low. Therefore, it was also assumed that the lack of diversity with regard to the kinetic parameters was the result of long-term selective pressure toward acquisition of lower and lower K_t values. Gradually, it was assumed, all the organisms would evolve the same low K_t (that is, the same high affinity for uptake). These assumptions are themselves based on the tacit assumption that an entire aquatic habitat is uniform with respect to concentration of any particular dissolved organic compound. They take no account of the potential heterogeneity that could exist within a water body on either a spatial or temporal basis. For selective pressure to result in only a single low K_t, for example, the organisms must never experience transiently high concentrations of substrate. If they did, one might assume that some of the organisms would have opted for exploitation of the "rich microzone" niche, and that this adaptation would be reflected in higher K_t and V_{max} values than those in organisms adapted to the ultra-low substrate concentrations of bulk water.

Microzones of organic enrichment do occur in natural waters. Such zones include the immediate vicinity of photosynthesizing phytoplankton (even those cells that are healthy), surfaces of suspended detrital particles, and the guts of aquatic animals. These regions might produce sustained high concentrations of dissolved organic substrates. In addition, transiently high concentrations might result from the fragmentation of organisms such as algae and protozoans when they are fed upon by zooplankton, and from the excretion of feces by aquatic animals. These considerations lead one logically to assume that the local concentrations of dissolved organic compounds in natural waters may at times greatly exceed the overall bulk concentration that would be measured after filtration (homogenization) of a water sample for organic analysis. Instead of monotonously low, nanomolar levels, the microheterotrophs might encounter, at various times, a wide range of substrate concentrations from nanomolar to perhaps many micromolar or even millimolar in enriched microzones.

RECENT FINDINGS

We have examined the uptake kinetics over a wide concentration range for a number of naturally occurring organic compounds by natural microheterotrophic populations in freshwater and marine environments (Azam and Hodson 1981; Kirchman and Hodson, unpublished). When the substrate concentration range used is increased to include concentrations that can be expected to occur in organically rich microzones and the data are treated in a manner similar to that of Wright and Hobbie (1966), straight line plots are rarely obtained. Instead, the data plotted on the "linearization" graph produce a hyperbola-like curve. No single K_t or V_{max} can be derived from the curves, but rather the curves suggest that a wide range of K_t and V_{max} values are represented in the population. Some components of the population appear to be adapted to using the ultra-low levels of organic substrate typical of bulk water, whereas other components are adapted to take advantage of very high concentrations where and when they might occur. The overall effect is the failure of the substrate uptake rate to saturate. The population as a whole can rapidly respond to a range of substrate concentrations that stretches over many orders of magnitude.

FUTURE RESEARCH NEEDED

Many pollutants are taken up (and subsequently degraded) by microorganisms because of the similarity of their molecular structure to naturally occurring substrates that the microbes are adapted to utilize. Models to describe the rate of removal of such compounds from natural waters have often assumed that the kinetics of uptake of the pollutant will reflect those of the natural analog. And the assumption for the natural compounds has previously been that they are removed via simple Michaelis-Menten kinetics by the natural mixed populations. These models predicted that uptake (removal) rates would saturate at some finite concentration of the compound in the water. What we now know about the multiphasic nature of uptake kinetics exhibited by many natural populations suggests that pollutant uptake rates might not saturate but continue to increase as higher and higher V_{max} uptake mechanisms come into play. At this time the presence or absence of multiphasic kinetics in the uptake of xenobiotic compounds has not been examined. However, the implications of the possible presence of this kinetic pattern with regard to modeling the fate of pollutants in aquatic environments warrant concentrating some research in this area.

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APPLICATION OF 3/2 ORDER KINETICS FOR SUBSTRATE BIODEGRADATION IN SOIL

Dennis D. Focht

ABSTRACT

The kinetics of mineralization of carbonaceous substrates is explained by a deterministic model which is applicable for either growth or non-growth conditions in soil. The mixed order nature (referred to as "3/2 order") does not require *a priori* decisions about reaction order, discontinuity periods of lag or stationary phases, or corrections for endogenous mineralization rates.

MODEL FORMULATION

The differential equation is

$$-\frac{dS}{dt} = K_1S + K^1SE \qquad (1)$$

When K^1E is expressed as a function of time we have

$$-\frac{dS}{dt} = K_1S + K_2St \qquad (2a)$$

for linear growth (i.e., $K'E = K_2t$) and

$$-\frac{dS}{dt} = K_1S + E_0e^{\mu t}S \qquad (2b)$$

for exponential growth, (i.e., $K'E = E_oe^{\mu t}$). The rate of CO_2 product formation

$$\frac{dP}{dt} = -\frac{dS}{dt} + K_o \qquad (3)$$

where K_o is the zero-order rate constant for mineralization of indigenous soil organic matter or, in the case of $^{14}CO_2$ evolution, the rate of mineralization from residual **product that has become part of the new humus. Upon integration of** equations 21, 2b, and 3 and by substitution we nave

$$P = S_o[1-e^{-K_1t - (K_2t^2)/2}] + K_ot \qquad (4a)$$

$$P = S_o[1-e^{-K_1t - \frac{E_o}{\mu}(e^{\mu t} - 1)}] + K_ot \qquad (4b)$$

for linear and exponential growth, respectively.

APPLICATION OF THE MODEL

CO_2 evolution data from soil were fit to the above equation by a nonlinear regression analysis with the "NLIN" program by SAS on an IBM 750 computer using the Marquardt algorith for stepwise iteration. This program gives a rapid convergence even when the initial estimates are considerably wrong. Linear growth (equation 2a) is applicable when initial cell densities are relatively high and do not increase more than 100 fold, while exponential growth (equation 2b) is applicable to low cell densities which increase by several orders of magnitude in response to substrate additions. Because the computer program might seem formidable and not accessible to non-mathematics, a linear transformation of equation 4a can be used after the values for S_0 and K_0 are determined by a simple linear regression analysis of the zero-order portion of the reaction (see Figure 1). The transformation to the linear form:

$$Y = -K_1 - K_2 t/2 \qquad (5a)$$

is then possible in which

$$Y = \frac{\ln[(So - P + K\ t)/S\ ]}{t} \qquad (5b)$$

This equation was found to be an adequate approximation for equation 4a if there are sufficient data points in the linear portion of the curve to determine S_0 and K_0 accurately. (It should be noted here that S_0 is not strictly defined as the initial substrate concentration, but rather the fraction of substrate that is readily converted to CO_2. This redefinition obviates the problem of accounting for substrate which goes into humus or biomass.

FUTURE RESEARCH NEEDS

The advantages of the 3/2 order kinetics to the Monod equation are as follows: (1) only 2 (K_1, K_2) rather than 4 (V_{max}, K_s, Y, X_0) interdependent constants have to be determined by nonlinear regression analysis (in lieu of equations 5a,b); (2) the required initial estimate can be easily obtained from a linearized form (equations 5a, b) rather than from an interval estimate of a differential equation; (3) substrate or product formation can be expressed as an explicit function of time; (4) biomass concentration does not have to be known.

The immediate question regarding the model is: what intrinsic characteristics do the rate constants possess? The zero-order constant K_0 represents the indigenous gross metabolic activity of the soil. Certainly this value will differ among soils, so it represents a base value: i.e., a soil with a higher K_0 would probably also effect more rapid degradation of added substrates than one with a lower K_0. Thus, S_0 the fraction of readily mineralized carbon would be dependent upon K_0. Compartive degradation rates of any given substrate might be made on this basis. In the absence of growth, the meaning of K_1, the "first-order" rate constant becomes very clear, and one would thus be justified in using a "half-life" expression for the degradation rate. The intrinsic meaning of K_2, the "second-order" rate constant, is not clear at this time. However, it is probaly more germaine to address the question of how one expresses the nonlinear rate process of a sigmoidal curve. Since we are concerned with expressing some quantitative meaning to the upper and lower rates of degradation, it is justifiable then to define the kinetic parameters Vm, the maximum rates of mineralization, and tm, the time at which this occurs, this can be determined by taking the second derivative of equation 2a to express the change in velocity.

$$\frac{d^2S}{dt^2} = K_1 \frac{dS}{dt} + K_2 t \frac{dS}{dt} + K_2 S = \frac{d^2P}{dt^2} \qquad (6)$$

and when $d^2S/dt^2 = 0$, $dS/dt = V_m$, so that

$$V_m = \frac{K_2 S_M}{K_1 + K_2 t_m} \qquad (7)$$

where S_m is the substrate concentration (i.e., the saturation constant)

$$t_m = \frac{-K_1 + \quad K_2}{K_2} \qquad (8)$$

This clearly states that $K_2 \quad K_1^2$; otherwise, $t_m \quad 0$, a situation that develops when there is no inflection point (i.e., the rate is essentially first order). Thus the t_m value is useful quantitiatively in telling us something about the length of the achieved.

When exponential growth gives a better fit than linear growth, the K_1 term has always been found to be negligible. Thus equations 2b and 4b are reduced to

$$\frac{-dS}{dt} = E_o \, e^{\ t} S \qquad (9)$$

$$P = S_o \quad 1 - e^{-E_o/ \quad (e^{\ t} - 1)} \quad + K_o t \qquad (10)$$

If we take the second derivative of equation 9 to find the inflection point as we did in the development of equations 6-8, we obtain for exponential growth

$$V_m = \frac{S_o \, e^{-E_o/}}{e} \qquad (11)$$

$$S_m = \frac{S_o e^{-E_o/}}{e} \qquad (12)$$

$$t_m = \frac{\ln (\ /E_o)}{\quad}$$

The application of the linear and exponential growth models to the mineralization of biphenyl in soil can be seen in Fig. 1 Linear growth gives a better fit with inoculation, while exponential growth gives a better fit in lieu of inoculation: presumably this is due to the low indigenous population density of biphenyl degraders.

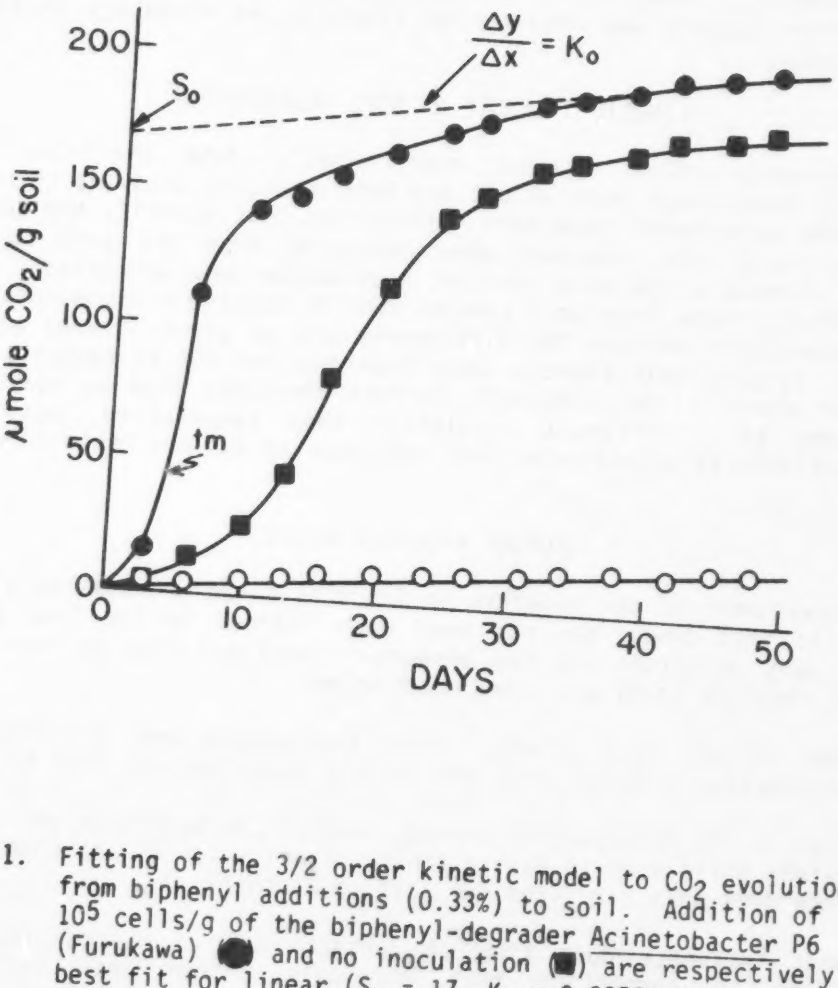

Fig. 1. Fitting of the 3/2 order kinetic model to CO_2 evolution from biphenyl additions (0.33%) to soil. Addition of 10^5 cells/g of the biphenyl-degrader *Acinetobacter* P6 (Furukawa) (●) and no inoculation (■) are respectively best fit for linear ($S_o = 17$, $K_1 = 0.0050$, $K_2 = 0.045$) and exponential ($S_o = 15$, $\mu = 0.18$, $E_o = 0.0059$) growth. The linear portion of both curves ($K_o = 0.090$) is the same as the control (○) without biphenyl. Complete mineralization of biphenyl would bring about 240 μmoles CO_2/g.

FACTORS CONTROLLING DEGRADATION RATES IN SOILS

D. A. Laskowski

ABSTRACT

In keeping with the workshop theme, this paper attempts to summarize the needs for characterizing degradation kinetics of chemicals in soils. Kinetics is becoming increasingly important because of its key role in any evaluation of environmental impact of chemicals. Modeling is definitely emerging as the premier tool for fate analysis and degradation kinetics are mandatory in such exercises.

KINETIC ANALYSIS OF SOIL DEGRADATION

Unfortunately there is not much kinetic data available on soil degradation, even though much effort has been spent in studying chemical fate in soil. Few experiments have been carried out that quantify the statistical variability in a rate constant when switching from one soil to another. Effects of climate on the rate constant have seldom been addressed. It is not known if kinetic data from soil can be used in aquatic environments and vice versa. Researchers believe the differences are so great between aquatic and terrestrial systems that kinetic data from one are not transportable to the other. But where is the evidence? Do rate constants from an aquatic system truly belong to a different population when temperature, moisture, and inherent variability within water and soil populations are factored out?

FUTURE RESEARCH NEEDS

Our assessment of the progress in learning about degradation kinetics of chemicals is that there has not been much learned in the last ten years. There are many questions but few answers. Questions that we ourselves have arrived at over the years are summarized below:

1. What effect does climate (soil temperature and moisture) have on degradation rate and does this effect vary from one soil to another?

2. What is the relationship between initial concentration and degradation rate? Soils seem to have a finite capacity to degrade chemicals. How much does this vary from one soil to another?

3. What influence does time of contact have on degradation kinetics? There is suggestion that chemicals become less available for degradation as residence time in soil increases.

4. Finally, how much variation is encountered in changing from one soil to another? How is this variation distributed statistically? Can it be reduced by factoring out correlations between rate and soil property data that can be obtained readily?

We embarked on a program to address some of these questions since it is not possible to model fate effectively without this information. Soils are being collected from across the country and will be used to examine relationships between degradation rate and the variables' initial concentration, soil temperature, soil moisture, and soil. We hope to have at least some answers from our experiments over the next couple of years.

PANEL REPORT

Environmental factors govern the rate and extent of biodegradation and, indeed, may determine whether or not biodegradation occurs. In addition, environmental factors determine the differences observed between biodegradation rates in laboratory tests and in the field, between different sites in natural ecosystems, and also at the same site but at different times. Nevertheless, inadequate information exists on which environmental factors in nature govern the rate and on the quantitative relationship between the ecologically significant factors and changes in rates. This information is essential for meaningful predictions of rates in natural ecosystems based on laboratory tests and of rates in different environments.

In some instances, there is general agreement on the importance of individual environmental factors, yet, even in these instances, the quantitative impact of increasing intensity of that factor on biodegradation rate is uncertain. In other instances only a general perception exists that a particular factor may be significant and, in these cases, the role of that factor must be established and then its quantitative impact must be established. Not all physical, chemical, and biological properties or characteristics of natural environments will have a significant effect, or any effect, on the rates of biodegradation. Yet widespread agreement does not exist on which factors should be ruled out as being unimportant.

Thus, we recommend that the environmental factors that are of primary importance in governing biodegradation rates be characterized. The factors of primary importance are: (a) temperature, (b) aeration, (c) concentration of xenobiotic, (d) history of prior exposure to xenobiotic or related chemicals, (e) salinity, (f) surfaces (primarily in flowing streams), (g) inorganic nutrients (primarily in aquatic environments), (h) moisture level of soils, and (i) soil and sediment type (including organic matter content, caution exchange capacity, texture, and clay type). Other factors that probably are of lesser importance or that are important under specific circumstances or for particular chemicals are (a) sorption, (b) suspended solids (aquatic systems), and (c) pH.

The significance of grazing on the biodegrading species by protozoa and other organisms, the importance of the sediment-water and air-water interfaces, and the use of rates of community metabolism as a predictor of biodegradation rates should also be considered. In each instance the quantitative relationship between the intensity of the factor and biodegradation should be established. Prime attention should be given to assessing which of these are the major factors affecting biodegradation rates, which are minor, and which have no detectable influence.

Representative chemicals should be chosen as benchmark compounds to be used in biodegradation studies. They should represent xenobiotics that intrinsically have different rates of biodegradation, and whose metabolism is initiated by markedly different types of enzyme reactions. These compounds should be chosen with care.

To facilitate the comparison of biodegradation rates under a variety of environmental conditions, we feel that a set of standard reference compounds (representative of various class of xenobiotics) be examined in a manner that produces easily comparable data. Using these various test compounds, we should compare controlled laboratory experiments to environmental studies. Individual factors should be manipulated in the laboratory and their effect on biodegradation assessed. The natural spatial and temporal variability in a geographically and trophically diverse set of systems should be exploited by using regression studies to correlate biodegradation with these factors.

Environmental factors may have different effects with different compounds. For example, the Q_{10} for the biodegradation of one compound may be 2, while the biodegradation of another may be 20, and thus they have different temperature coefficients. Or, with respect to aeration, the rate of hydrocarbon degradation would be drastically affected (from relative rates of 100% to 0% while the rate of glucose degradation would be relatively unchanged, (e.g., 100-75%) by varying aeration status. Hence, we recommend that research on the role of environmental factors be designed to evaluate their impacts on compounds whose biodegradation will probably be affected differently or to different extents by changes in the same factor.

The confidence intervals for the quantitative effects of each of these environmental factors must be defined. Then, the significance of each of these factors and the interactions among them must be validated by field trials. If these field trials suggest that factors other than those proposed here are important, these factors should be defined and their quantitative impact evaluated.

The apparent lag phase or period for acclimation is important because it may be a significant part of the time that the compound persists. Yet, almost nothing is known of factors affecting the length of this phase or its cause (i.e., time for population growth, induction or enzymes, or genetic changes). Therefore, research is needed to establish these factors and to determine the reason for this apparent lag phase.

APPLICATION OF UPTAKE AND MINERALIZATION KINETICS

Objective: To evaluate the potential application of uptake and mineralization kinetics to the biodegradation of xenobiotic chemicals by natural microbial communities.

The biological transformation of naturally occurring organic materials at concentrations typically found in receiving waters or soil has been extensively studied by kinetic analysis of the rates at which radiolabeled substrates are taken up and/or mineralized to carbon dioxide by heterogeneous microbial communities. These studies are based on the demonstration of saturation-type kinetics from which estimations of specific and maximum turnover rates, half-saturation constants, and specific activity indices can be derived. Only recently has this kinetic approach been applied to the transformation of xenobiotic chemicals. Is this a reasonable application, and what new information or insights can we expect to gain from this approach which cannot be obtained by other approaches? Can saturation kinetics be demonstrated for most xenobiotic chemicals? Can specific activity indices be used as a method for estimating the fraction of the microbial population involved in biodegradation? Must xenobiotic chemicals be used as carbon and/or energy sources by microorganisms before these kinetic techniques can be applied?

Panel Members: Carol Litchfield, Dupont
Fred Pfaender, University of North Carolina
Bob Larson, Proctor and Gamble
Don Button, University of Alaska
Roy Ventullo, University of Dayton

HISTORICAL BACKGROUND TO THE MEASUREMENT OF HETEROTROPHIC POTENTIAL IN THE AQUATIC ENVIRONMENT

C. D. Litchfield

ABSTRACT

My purpose here is to present an historical background to the measurement of heterotrophic activity in the aquatic environment. This will enable all of us to have the same frame of reference for evaluating the application of this technique to biodegradation studies. These applications will be described by the other members of this panel.

HISTORICAL BACKGROUND

In 1951 Steeman-Nielsen published the first paper on the use of ^{14}C labeled carbon to estimate the production of organic matter in the ocean by phytoplankton (5). The next year he applied this method to field studies and described the technique which is still used, essentially unchanged since that paper was published (6). In 1962 Parsons and Strickland applied the radiocarbon labeled concept to the measurement of heterotrophic processes using ^{14}C-labeled organic substrates. They noted that a Hanes transformation plot (1) of the data derived using Michaelis-Menten kinetics usually resulted in a straight line. From the slope and intercepts of that line they then calculated their estimations of heterotrophic potential (4). In that paper, however, they cautioned, "the method is rapid and convenient and can give values of relative heterotrophic potential. As with the radiochemical method for measuring marine photosynthesis, the exact interpretation of results presents many problems...." (4) (the emphases are mine).

As useful as the procedure was, they did not recognize, however, that the majority of the carbon was not converted to biomass. In fact, it is not unusual for the percent converted to biomass to account for only 10-30% of the total carbon used. By 1966 it had become apparent that a major correction was needed to account for the respired CO_2, and such corrections would obviously be important to any interpretation of heterotrophic potential. Two different approaches to solving this problem were presented by Kadoata and his co-workers (3) and by Williams and Askew (7). These papers provided the basis for the publication in 1969 by Hobbie and Crawford of their paper, "Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters" (2).

With this newer information, then, the procedure began to be widely applied in microbial ecological studies, and numerous papers have been published discussing the assumptions, applications of the technique to different ecosystems, and the problems with the interpretation of heterotrophic uptake/mineralization studies.

REVIEW OF THE BASIC TECHNIQUE

I would now like to review very briefly the basic technique used in measuring heterotrophic activity, the calculations which are used to derive "heterotrophic potential," and the assumptions inherent or implied in adapting Michaelis-Menten kinetics to ecological studies. Typically the tests are performed in an enclosed sample bottle where the respired CO_2 is trapped on a suspended piece of filter paper moistened with phenethylamine or KOH. Determination of the amount of $^{14}CO_2$ respired is performed using a liquid scintillation counter and the resulting data are used to derive the estimates of heterotrophic mineralization. To determine the heterotrophic uptake, the incubation mixture is filtered, washed, and the filter also counted. The amount of radiolabeled carbon in or on the cells is the basis for the calculations of heterotrophic uptake. Several concentrations of the substrate and several time periods (usually five minutes to three hours or less) are necessary unless one already knows the _in situ_ concentrations of the substrate and the biomass of organisms able to utilize that particular substrate.

There are many assumptions inherent in applying Michaelis-Menten kinetics to such studies:

- The substrate is present at saturating concentrations.
- There is no significant change in the substrate concentration during the incubation period.
- There is no change in the concentration of the biomass of the actively metabolizing population.
- There are no activators required nor inhibitors present.
- In the equation below, K_3 is the rate limiting step:

$$\text{Bacteria (B)} + \text{Substrate (S)} \quad \underset{K_2}{\overset{K_1}{\longleftrightarrow}} \text{BS} \xrightarrow{K_3} \text{B} + \text{Product(s)(P)}$$

Although not part of the kinetic assumptions, other requirements in the mechanics of these studies are that: samples must be incubated at _in situ_ temperatures and pressures, if applicable; added substrate concentrations _must_ be less than the background or natural substrate level; and the oxygen, redox, and pH conditions should approximate _in situ_ conditions.

If all of these assumptions and conditions have been met, the data can then be plotted using the equation:

$$t/f = \frac{1(S)}{V_{max}} + \frac{K_t + S_n}{V_{max}}$$

The resulting linear plot is shown in Figure 1 where t/f is plotted against the added substrate concentration, and the slope of the line is $1/V_{max}$, the y-intercept is a function of the _in situ_ substrate concentration plus an indication of the affinity of that population for the substrate (i.e., a high K_t will require a high substrate concentration to activate the microbial population).

If all of the conditions and assumptions have not been met, then it is invalid to apply Michaelis-Menten kinetic equations to the analysis of the data.

FUTURE RESEARCH NEEDS

These types of studies can be performed using substrates not commonly found in the environment, xenobiotics, or even naturally occurring organic compounds. We have been tracking changes in the heterotrophic mineralization rates for several naturally occurring materials. The results with urea are shown in Figure 2 where the amount of urea C mineralized per 24 hours is plotted for different zones in the New York Bight apex. Using the heterotrophic mineralization/potential approach, then, we are able to estimate or compare relative microbial activities in different marine environments over a prolonged time.

If the investigator remembers the limitations of this method and applies the resulting data appropriately, heterotrophic potential measurements can provide a relatively rapid indication of the ability of the existing microbial population to transform and metabolize organic compounds entering the environment.

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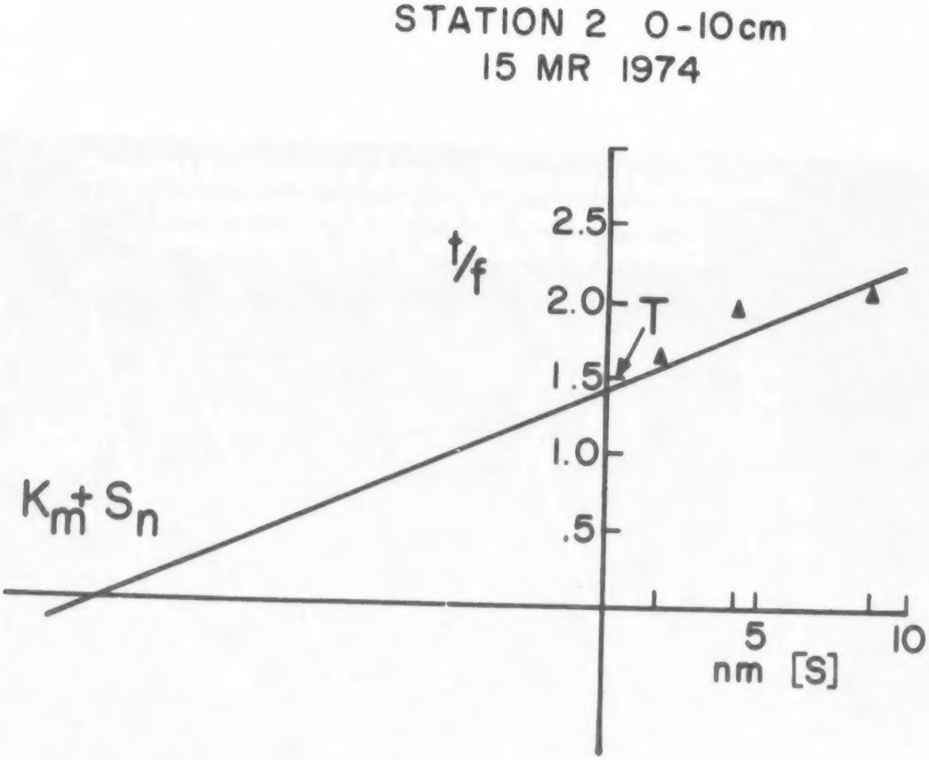

Figure 1. Typical Hanes plot of ^{14}C-mineralization data. The plot shown is for one location in the 0-10 cm portion of a core from the sediments at Sandy Hook Bay, New York.

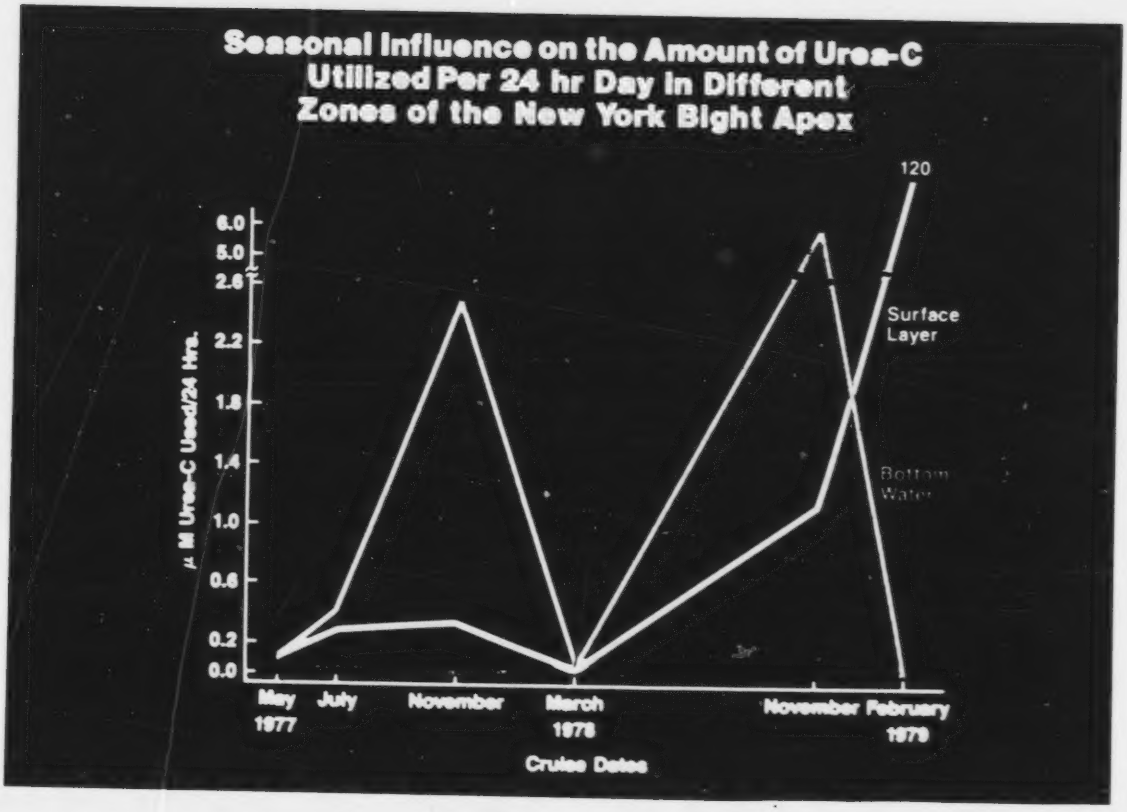

Figure 2. Seasonal influence on the amount of Urea-C utilized per 24 hr day in different zones of the New York Bight Apex.

APPLICATION OF UPTAKE AND MINERALIZATION KINETICS

Frederic K. Pfaender

ABSTRACT

Over the last several years we have been investigating the application of heterotrophic uptake kinetics to the measurement of pollutant biodegradation. Various versions of the technique have been used, with varying degrees of success, for many years to assess the heterotrophic potential of natural aquatic bacteria, using substrates like glucose, acetate, and amino acids. The approach is based on Michaelis-Menten kinetics, and was developed to describe the kinetics of enzyme-substrate reactions. The application of this approach to mixed populations of intact microorganisms has often been questioned. **The saturation response has been shown empirically in many, but not all, environments and** for a variety of substrates. **The fact that saturation is obtained for many pollutants is taken as an indication that this kind of kinetic approach can be applied to describing the activities of microorganisms in environmental samples.**

APPLICATION OF MICHAELIS-MENTEN KINETICS TO POLLUTANT DEGRADATION

The use of Michaelis-Menten kinetics to describe pollutant biodegradation rates requires that certain assumptions be made:

1. For the measured kinetic parameters to be accurate, the amount of enzyme present during the incubation period must remain constant. The extension of this assumption to environmental samples means that the numbers of organisms and/or the amount of biomass should remain constant during the incubation. This, of necessity, will require a relatively short incubation period, since it is reasonably well known that the numbers of organisms do change while confined in a container.

2. It is also required that the concentration of substrate not change significantly during the course of the measurement. These two assumptions can only be satisfied if the measurement is made over a relatively short period of time. Long-term incubations will result in both an increase in the number of organisms present and a decrease in the concentration of the substrate, due to its breakdown by the microorganisms.

3. The assumption is made that the transport systems present are responding only to the compound added as substrate, and not to other compounds present in the environment.

4. In our particular case, we make the assumption that concentration of the specific pollutant substrate present in the environmental sample is negligible compared to the amount we add as substrate. This assumption allows us to disregard the natural substrate concentration in the calculation of the kinetic parameters.

We feel that the use of this kinetic approach to measuring pollutant biodegradation has several very positive features. From past experience in our laboratory, we know that confining samples within a bottle results in significant changes in both the size and activity of the microbial community. We also know that there is a period of approximately 12 to 16 hours before these changes become significant. Therefore, if biodegradation rates can be measured during this time period, a rate can be generated that might be quite close to that which would occur under natural environmental conditions. Since the assumptions required for use of this technique necessitate short-term incubations, this methodology appears quite appropriate for answering questions about real world rates. In addition, the kinetic approach offers the advantage of being able to calculate several useful biodegradation parameters. These include V_{max}, the maximum potential velocity that the indigenous community can attain, Km or the half-saturation constant which gives an indication of the concentration range to which the community is adapted. In addition, a rate constant, which we have called K_1 and has the same units as a first-order rate constant, can be calculated from the kinetic data in several different ways. These include the dividing of V_{max} by Km, or taking the slope of the linear portion of the velocity vs. concentration plot. Two additional non-Michaelis-Menton parameters can be calculated from the data generated in these experiments. The velocity of metabolism with any particular added concentration can be obtained from the data generated by multiplying the concentration added by the f/t factor. In addition, the turnover time for the added xenobiotic at any particular concentration can be measured directly from the linearization of the kinetic data.

INTERPRETATION OF EXPERIMENTAL RESULTS

We can calculate several parameters from the uptake and mineralization data, but have questions about which one is most useful. V_{max} may represent the maximum potential velocity, and when we look at the saturation plots we see that for some compounds saturation occurs at what may be environmentally realistic concentrations (cresol Figure 1, and chlorobenzene Figure 2). For other compounds, however, (NTA) many hundreds of mg/l are needed, which are concentrations far in excess in what might actually occur. We began calculating K_1 because it is derived from the slope of the linear portion of the curve, generally in the lower part of the concentration range. We felt that it gave a realistic additional measure of biodegradation. When we do correlations of biodegradation rate measures and environmental characteristics (Table 1), V_{max} gives relationships that make sense. In coastal environments where nutrient concentrations are low, we get a strong relationship with nitrogen and phosphorous species and chlorophyll, which is the principal source of nutrients. In the lake and river environments where concentrations of nutrients are many times higher than the coastal environments, nitrogen and phosphorous do not appear to be strongly related to biodegradation rates. Although not shown in the table, when K_1 and V_{max} are correlated with one another and with environmental parameters, they generally correlate well with one another and with the same characteristics of the environment. While it is reassuring that these two measures of biodegradation appear to be describing similar processes, it does not help a great deal in determining which one gives the most realistic or useful measure of biodegradation. Since both are obtainable from the same set of laboratory measurements, it is always possible to calculate and report both.

POTENTIAL FOR OBSERVING SATURATION KINETICS

We have always been able to obtain saturation if appropriate concentrations are used. As mentioned previously, for some compounds this requires higher concentrations than are likely to occur in the environment. We are also aware of several reports in the literature of saturation not being obtained with many natural substrates. Since we have looked at only four or five pollutant compounds, in only a few environments, it is difficult to make generalizations about many different classes of materials. This raises questions about the conditions under which saturation may be obtained. In attempting to shed light on this question, we have used several techniques to estimate the number of degraders of specific compounds. Table 2 shows the results of this evaluation in some fresh water samples using two methods, plate counting and microautoradiography. The important point of this table is that the specific degraders constitute a relatively small part of the total community. I believe the reason we are able to obtain saturation is because this small community behaves in a manner more similar to a pure enzyme system than would the larger, more diverse community that might be metabolizers of naturally occurring substrates. Since the technique we use was developed to measure the activity of pure enzyme systems, I do not consider it surprising that we are able to obtain saturation when working with a very small part of the total assemblage of microorganisms present in an environmental sample. These questions, however, are still far from being answered.

EFFECTS OF INCUBATION PERIODS

The uptake and mineralization technique that we have used to assess pollutant biodegradation requires that the measurements be made over a relatively short period of time. During this short incubation period only a small proportion of the label added is metabolized, usually from one to three or four percent. This obviously raises the question of whether this short-term rate can be extrapolated to the longer periods of time necessary to achieve complete breakdown of the pollutant. In an attempt to gain some understanding of this we have conducted time course experiments in which extended incubation periods were used and the amount metabolized over time measured. Table 3 shows the results of this study for two compounds, chlorobenzene and meta-cresol. We have used K_1 to predict the percent of the compound that would be degraded at different time periods and compared this to what was observed in the time course study. We have also used V_{max} to predict how many micrograms of the material would have been metabolized at the same time periods and compared this with the micrograms actually converted to CO_2 and cells. As you can see, for both compounds and both methods of estimating biodegradation, there is good agreement between the two methods for an initial period of several days. However, after five to seven days confinement in the time course studies, the amount of degradation slows down, yielding an apparent underestimation of the amount of pollutant that would be biodegraded, as predicted by the kinetic approach. It is interesting to note the good agreement between the V_{max} prediction and the micrograms actually metabolized. This would tend to indicate that in the containers used for the time course study the community is functioning at close to V_{max}. Since we do not have information on the long-term degradation of these compounds in the environment tested, we have no way to determine which method is providing the most accurate estimate of persistence. However, good agreement between the two techniques during the early stages of incubation and the fact that changes are known to occur in the container after some period of confinement would tend to point to the kinetic approach, giving a more realistic estimate of the amount of time necessary for biodegradation.

FUTURE RESEARCH NEEDS

We are relatively confident that the uptake and mineralization approach has potential as a rapid and useful measure of pollutant biodegradation. However, additional research needs to be addressed to several pertinent questions:

1. To how broad a range of compound and environment can this approach be successfully applied? At this point we have examined four or five compounds in three coastal and four freshwater environments. These sample sites are all in one geographic part of the United States, and certainly do not represent the range of environmental characteristics that might be found throughout even the United States.

2. Under what environmental conditions does saturation occur? While we know that it occurs for the compounds and environments we have tested, neither represent the range of materials or sites that are available. The methodology offers the opportunity to calculate several degradation parameters (K_1, specific velocities and turnover times) even if saturation is not obtained.

3. How valid are extrapolations based on the uptake of only a small part of the added compound? In the comparisons we have done with time-course studies, the extrapolations looked to be reasonably good, at least for a period of several days. One of the problems we face in testing these extrapolations is in determining what to compare the extrapolations to. While time-course studies are extremely useful in determining whether a compound is degradable and required for identifying degradation products and mechanisms, their use for estimating environmental rates of biodegradation appear questionable, but there are few other ways to do comparisons.

4. Does adaptation occur at the low environmental concentrations of most pollutants? This is a question that relates not only to this technique, but to all of the methods that we are discussing. What we really need to know is whether the low concentrations of those pollutants that we find in most environments constitute a selective pressure for the community of microorganisms that are present. This raises additional questions of how do we measure adaptation and what factors in the environment might be influencing whether or not organisms can adapt.

5. Why do metabolic rates change when natural communities are confined? Are the observed changes in rate due to alterations in the biomass present, alterations in the species composition or organisms present, or the result of changes in substrate concentration that result from microbial activity.

6. Is the assumption that the microbes are responding to only the added substrate valid? This question is important for an evaluation of the uptake and mineralization technique, since one of the assumptions we make is that the organisms respond only to the compound added. I think, however, this question has broader interest in terms of evaluating the specificity of the enzymatic mechanisms available for the degradation of pollutants. It is quite well known from laboratory studies that many organisms have transport systems that function with several different substrates. In environments where nutrients are scarce, it would be to the organism's advantage to be able to utilize a range of compounds with as few enzymes as possible. In our particular case, this is significant because the kinetics may be influenced by not only the substrate we add, but by other compounds that may be present in the environmental sample.

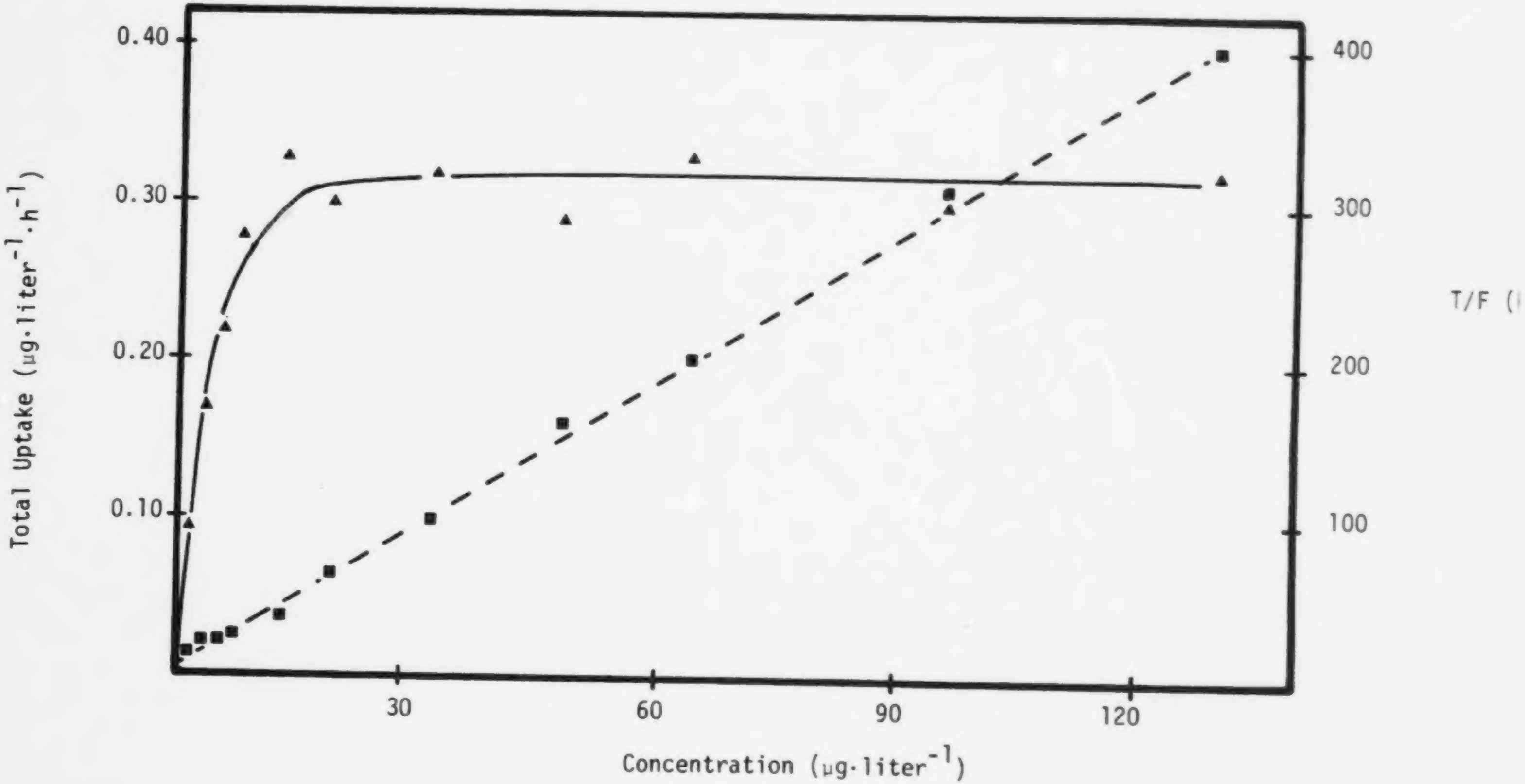

Figure 1. M-cresol uptake by Jordan Lake microbial community on 6/22/82, (▲—▲) - velocity, (■—■) - linear transformation.

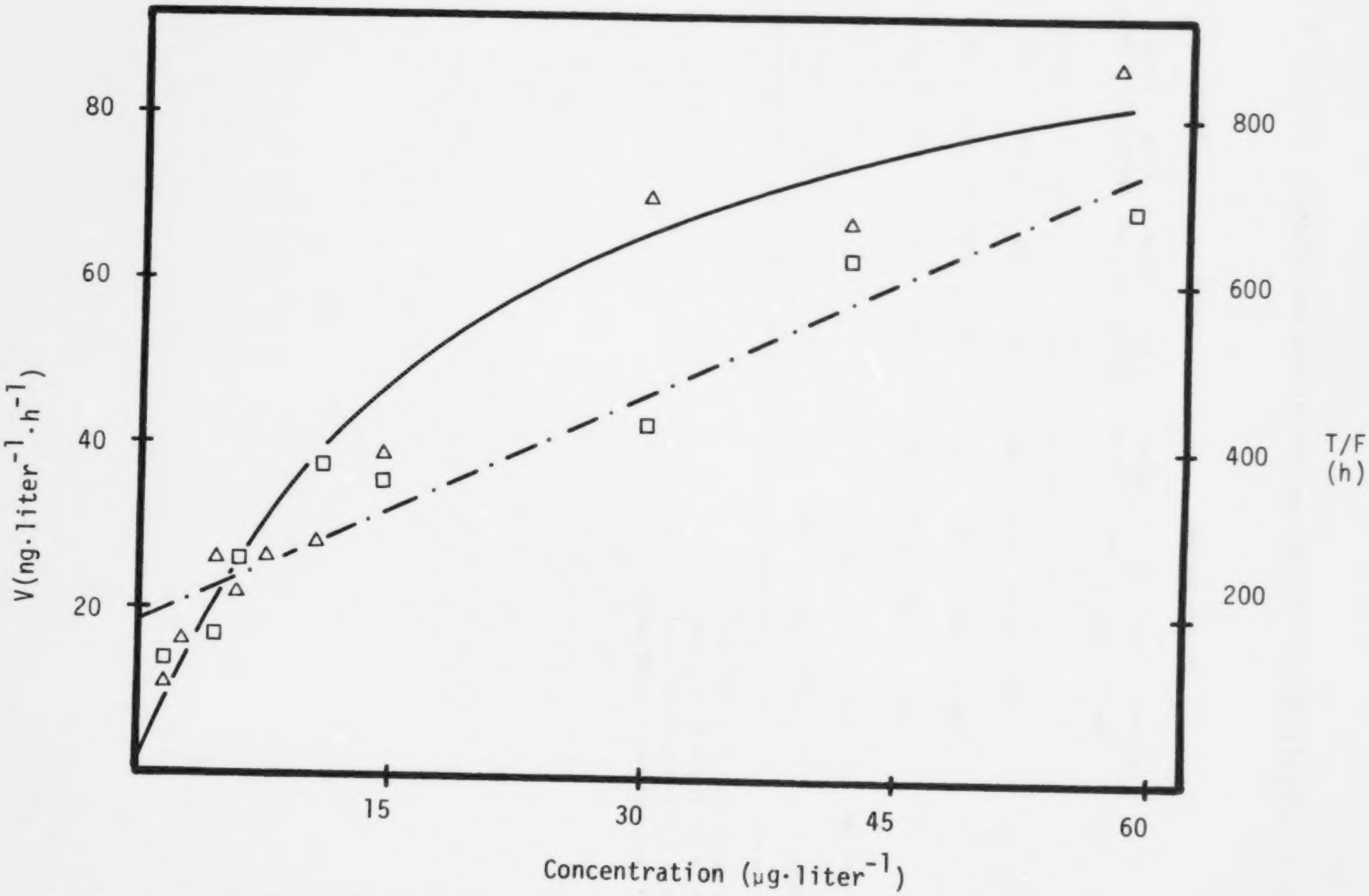

Figure 2. Total uptake of chlorobenzene by Lake Michie microbial community on 11/2/82, (△—△) - velocity, (□·□) - linear transformation.

Table 1. Correlation of nutrient concentrations and metabolic parameters

	Coastal Systems				Lakes		Rivers	
	m-Cresol V_{max}	NTA V_{max}	% Max Uptake	Amino Acid Het	% Max Uptake	Amino Acid Het	% Max Uptake	Amino Acid Het
NO_3 + NO_3-N	.82***	.81**	.76***	-.08	.23	-.36	-.18	.07
NH_4	.69**	.44	.59*	-.22	.49	-.36	.11	.06
PO_4	.74**	.80**	.67**	-.16	.49	-.39	.16	.01
Tot Part. Wt.	.08	-.11	.01	-.19	-	-	-	-
Chlorophyll a	.85***	.06	.62**	-.20	-	-	-	-

+ = Correlated at 90% level
* = Correlated at 95% level
** = Correlated at 99% level
*** = Correlated at 99.9% level

Table 2. Enumeration of specific degraders for m-Cresol in North Carolina rivers and lakes

Characteristic	Sample Source Haw	 Eno	 Michie	 Jordan
Total heterotrophs ($CFU \times 10^4/ml$)	23.0	14.5	13.8	9.5
m-Cresol degraders ($CFU \times 10^2/ml$)	3.30	1.36	.30	4.0
% m-Cresol degraders	.14	.09	.02	.42
Acridine Orange Direct Count ($\times 10^6/ml$)	4.3	5.7	8.2	16.0
m-Cresol degraders microautoradiography ($\times 10^4/ml$)	18.5	25.0	30.1	14.3
% m-Cresol degraders	4.3	4.3	3.7	0.89

Table 3. Comparison of persistence predicted by heterotrophic uptake and time course studies with Jordan Lake

Compound	Time (days)	% Metabolized		ug Metabolized	
		K_1 Prediction	Observed	V_{max} Prediction	Observed
Chlorobenzene	1	5.5	3.5-6	.033	.03- .07
	4	22.1	18-39	.134	.06-.186
	7	38.6	41-52	.235	.09-.237
	14	77.2	32-57	.470	.12-.175
m-Cresol	.5	8.4	.5-8	.09	.037-.087
	1	16.8	2-16	.18	.082-.21
	4	67.2	50-62	.72	.34-5.6
	7	117.2	45-62	1.26	.34-3.7
	14	235	45-62	2.52	.34-3.87

HYDROCARBON BIODEGRADATION: KINETIC CONSTANTS AND THEIR APPLICATION

D.K. Button, K.S. Craig and B.R. Robertson

We have examined the persistence of hydrocarbons in aquatic environments with focus on those hydrocarbons in the dissolved phase as they are transformed and biodegraded by bacteria. Our main experimental approach has been to follow radiolabeled hydrocarbons through both laboratory and environmental systems with data interpreted from biochemical and kinetic points of view. Our main interest is in the biological principles of hydrocarbon transport mechanisms and kinetics.

Early work led to the development of a sensitive technique for following the rate of CO_2 liberation from hydrocarbons (Button et al., 1981a). We were then able to locate sites of high degradative activity. Rates were fast in Port Aransas, Texas, near industrialized Corpus Christi (manuscript in preparation). In Port Valdez, Alaska, rates were even faster in a thin layer at 50 m depth due to ballast water discharge (Button, et al., 1981b).

In Resurrection Bay, Alaska, rates of toluene metabolism were initially slow, but could be induced to higher values, perhaps due to a microflora subsisting on conifer-derived terpenes (Button, Appl. Environ. Microbiol., in press). By exposing seawater samples to a range of toluene concentrations, we found that induction proceeded at its maximal rate after 48 h and at half its maximal rate (K_{ind}) at a substrate concentration of 1.9 μg per liter (manuscript in preparation).

Microautoradiographic techniques, together with epifluorescence microscopy, gave the original population of toluene oxidizers in this pristine fjord. About 8% of the bacteria (27 μg/liter) had the capacity to metabolize toluene. The process of induction raised the specific affinity for toluene (a_A) of this natural population from a base of 4.2 liters/g cells x hours to 11.6.

Induction raised the specific affinity of the marine isolate Pseudomonas T2 from a base of 0.03 to 500. The saturation constants k_T for natural assemblages of bacteria in seawater and for Ps. T2 were found to have values of 2 and 44 μg/liter, respectively, about the same as the concentrations that gave half-maximal rates of induction, K_{ind}. This similarity between K_T and K_{ind} is explained if toluene metabolism proceeds according to constitutive systems with the above values of K_T and then induces additional enzyme (such as toluene dioxygenase) at the rate-limiting step. Then induction and transport are controlled by the same process. A peculiarity of this system is that while the values for specific affinity are quite usual, the values of K_T are very small (manuscripts in preparation).

The formation of large quantities of metabolic products (type compounds shown in Fig. 1) appears to be a phenomenon only of very dilute microbial populations (Fig. 2), such as those in seawater. The recycling step of product reaccumulation by the bacteria is subject to different types of transport systems, perhaps of different classes (see below) and certainly with different kinetics. This phenomenon of dilute solutions necessitates a correction in the basic assumption that the kinetic constants for biodegradation are biomass-independent. The biomass dependency can be demonstrated by numerically simulating steady state systems using a continuous culture model and solving for biodegradation rates using the kinetic constants for toluene utilization, together with the product formation and re-utilization kinetics determined from 3-methylcatechol metabolism experiments.

Basic formulations for biodegradation kinetics and their relation to Monod kinetics are shown in Fig. 3 (Button, 1983). Also shown is the relationship between the kinetics and enzyme content of the organism at the rate limiting step.

Consideration of these kinetics has led to a model for the active transport of hydrocarbons called vectorial partitioning. It is only the fourth transport mechanism known and is shown in Fig. 4. Recent inhibitor data support this mechanism.

The understanding of biodegradation in aquatic systems has increased dramatically over the last few years. Four areas in need of additional work are:

1) Concentration-dependent kinetics and specificity of induction, particularly the extent to which hydrocarbons induce low-level metabolic activity in the bulk of the natural aquatic microflora when the inducers are sustained at very low concentrations over long periods. These data are useful because they reflect the concentrations of a pollutant which aquatic organisms are experiencing but which are rather difficult to measure. Also data define the concentrations of one hydrocarbon that would be expected to assist in priming a system for the biodegradation of another.

2) The nature and kinetics of ampholite degradation. While apparent Michaelis constants for the transport of hydrocarbons seem to differ radically from those for oxygenated substrates due to different mechanisms of accumulation, many anthropogenic hydrocarbons as well as their products of biodegradation are only mildly polar. Little is known of the decomposition kinetics of these compounds as they move from recognition by non-polar transport mechanisms to being accumulated by transport systems for polar substrates. For example, if an ampholite is accumulated by vectorial partitioning, it may be of little use to study its degradation at concentrations above nanomolar levels.

3) The chemical nature of organic products formed from hydrocarbon biodegradation. Apparently hydrocarbon biodegradation, as studied in the laboratory, contains metabolic loops which proceed outside the cell. In natural aquatic environments the biomass is too low for product reaccumulation to occur, so these products are lost, generating a range of unusual organic chemicals which can only be biodegraded with what may be rather low-affinity transport mechanisms.

4) The mechanism for active transport of non-polar substrates. If vectorial partitioning is the main mechanism for hydrocarbon transport, knowledge of this mechanism is useful for accurate anticipation of how the process will proceed for new compounds without having to test each alone.

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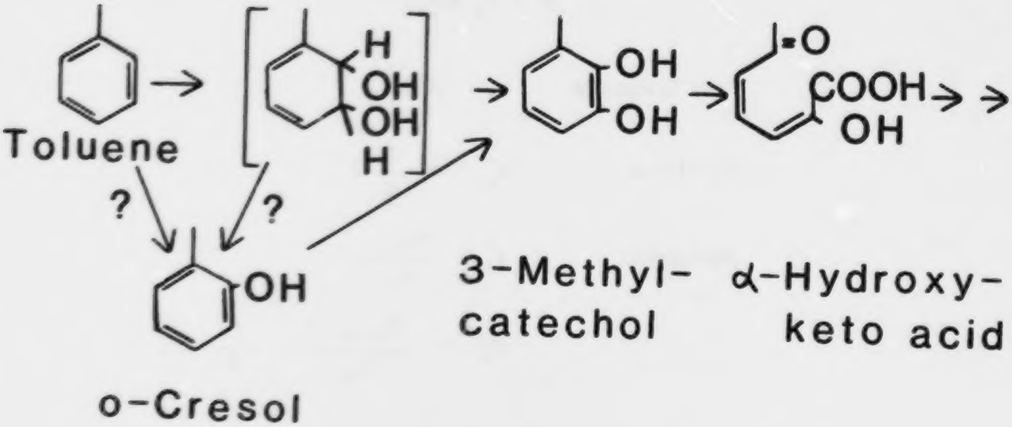
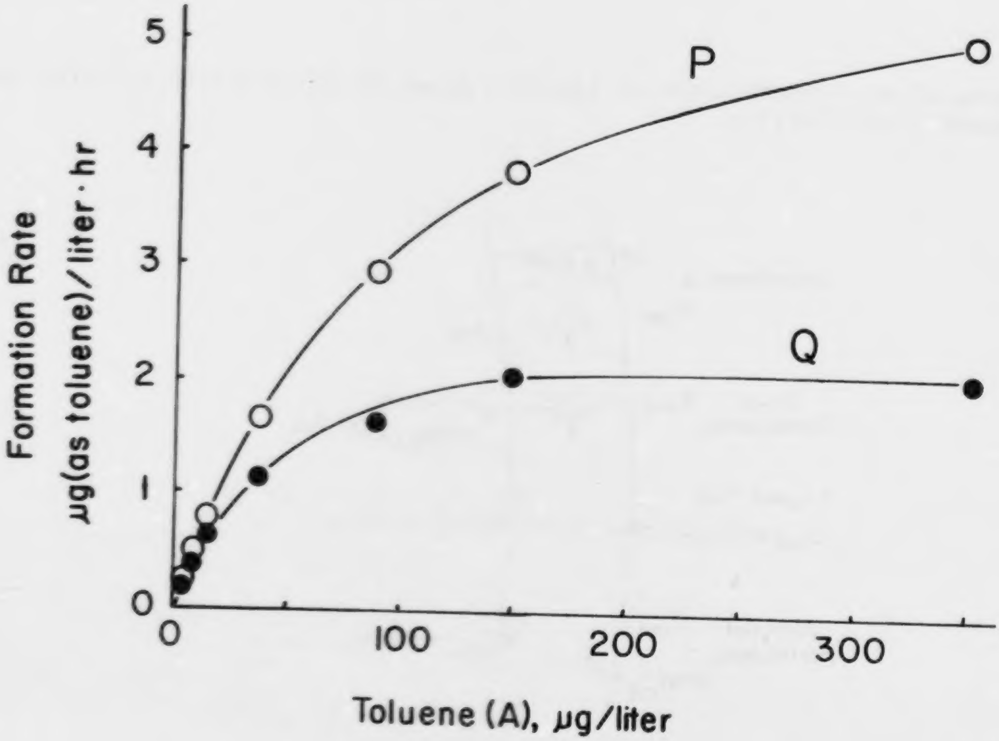
Button, D.K., Trends Biochem. Sciences 8, 121-124 (1983).


Toluene
?
?
o-Cresol
OH
H
OH
OH
H
OH
OH
O
COOH
OH
3-Methyl-catechol
α-Hydroxy-keto acid

1. Chemical nature of products formed from toluene by Pseudomonas T2.



2. Formation of organic products (P) and carbon dioxide (Q) as a function of initial toluene concentration by Pseudomonas T2.

$$v = a_A^{\circ} XA$$

Polar Substrate $\quad a_A^{\circ} = k \dfrac{T}{X}$

Non Polar $\quad a_A^{\circ} = k \dfrac{E}{X} \xi$

Michaelian $\quad a_A^{\circ} = \dfrac{V_{max}}{K_T}$

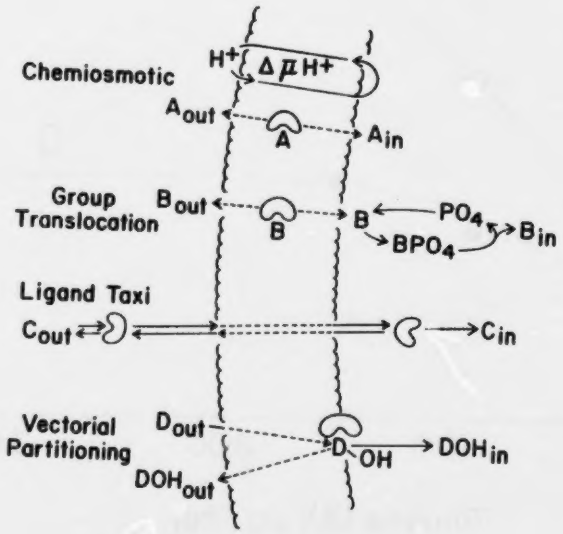
$$a_A^{[\,]} = \frac{V_{max} - v}{K_T} \; \frac{1}{X}$$

Monod $\quad \mu_{max} = V_{max}$

$$K_{\mu} = K_T$$

$$Y = \text{constant}$$

3. Formulation of biodegradation kinetics based on the specific affinity and second order kinetics.


Chemiosmotic
H^+
$\Delta \bar{\mu} H^+$
A_{out}
A
A_{in}
Group Translocation
B_{out}
B
B
PO_4
B_{in}
BPO_4
Ligand Taxi
C_{out}
C_{in}
Vectorial Partitioning
D_{out}
D
OH
DOH_{in}
DOH_{out}

4. Model for transport of hydrocarbons into bacteria by vectorial partitioning together with other models for active transport.

A HETEROTROPHIC BIODEGRADATION POTENTIAL (HBP) APPROACH FOR PREDICTING BIODEGRADATION RATES IN NATURAL ECOSYSTEMS

Robert J. Larson

ABSTRACT

Numberous investigtors (1, 4, 5, 10, 12-14) have shown that the rate of uptake and/or mineralization of radiolabeled natural substrates in aquatic systems can be described by a hyberbolic enzyme-saturation model of the Michaelis-Menten type (9). Recently, saturation kinetic models have also been applied to biodegradation of various xenobiotic compounds in ground and surface water systems (6, 11). The application of saturation models to xenobiotic biodegradation has important implications for environmental fate research. These models directly relate the rate of biodegradation of a specified substrate to the activity of the degrading microbial population. As such, they may be useful in normalizing microbial numbers for microbial activity and extrapolating biodegradation rate data to different environmental systems.

THEORY

The general equation relating the rate of uptake (and/or mineralization) of a ^{14}C-labeled substrate to the concentration of added substrate (when the background substrate concentration is unknown) can be written as:

$$v = \frac{V_{max}(S_b + A)}{K + (S_b + A)} \qquad [1]$$

where v is the degradation rate (ng/liter/h), V_{max} is the maximum degradation rate (ng/liter/h), S_b is the background substrate concentration (μg/liter), A is the added substrate concentration (μg/liter) and K is the half-saturation constant where $v = 0.5\ V_{max}$ (μg/liter). When the initial degradation rate is constant, v can also be defined as:

$$v = \frac{f}{t}(S_b + A) \qquad [2]$$

where f is the fraction taken up and/or mineralized at time t (h). Traditionally, equations [1] and [2] have been combined, inverted, and rewritten as follows to estimate kinetic constants (8):

$$\frac{f}{t}(S_b + A) = \frac{V_{max}(S_b + A)}{K + (S_b + A)} \qquad [3]$$

$$\frac{f}{t} = \frac{V_{max}}{(K + S_b) + A} \qquad [4]$$

$$\frac{t}{f} = 1/V_{max}(A) + \frac{(K + S_b)}{V_{max}} \quad [5]$$

A plot of t/f vs. A (equation [5]) yields a straight line with y-intercept T_n, x-intercept $(K + S_b)$, and a slope of $1/V_{max}$ (Figure 1). T_n is defined as the time required for complete degradation of a chemical at its in situ concentration, V_{max} is the extrapolated maximum degradation rate at infinite substrate concentration, and $(K + S_b)$ represents the sum of the half saturation constant and the background substrate concentration. Although unweighted linear regression of t/f vs. A (equation [5]) is commonly used to estimate kinetic constants, direct nonlinear regression analysis of the uninverted equation (equation [4]) can also be used to estimate these constants. Nonlinear techniques offer special advantages over standard linear techniques, in that they avoid many of the statistical biases inherent in linear transformations of nonlinear data (8). They also give better estimates of kinetic constants than linear techniques and are readily available to anyone with access to a microcomputer.

Equations [4] and [5] have traditionally been used to determine kinetic constants when $A > S_b$. A special situation occurs, however, when S_b is either much greater than A or so low as to be considered negligible. When $S_b >> A$, turnover times can be calculated directly from the ratio t/f using a single concentration of A. The concentration of added substrate is so low that it does not influence the in situ degradation rate (3), and observed T_n values represent the actual turnover time for the background level of substrate present. Since only one substrate concentration is tested by this technique, however, V_{max} and $K + S_b$ values cannot be calculated. When $S_b << A$, as would be the case when testing xenobiotics in previously unexposed systems, S_b can be considered zero for all practical purposes and ignored when combining [1] and [2]. Under these conditions, the following equation can be written:

$$v = \frac{f}{t} \cdot A = \frac{V_{max} \cdot A}{K' = A} \qquad [6]$$

where the half saturation constant is written as K' to differentiate it from the half saturation constant (K) of equation [5] which includes an S_b term. Strictly speaking, turnover times have no meaning when $S_b \sim 0$. Therefore, in systems where the in situ concentration of xenobiotic is negligible, T_n values cannot be calculated, although a ratio t/f may be obtained. However, equation [6] can be used to determine the empirical kinetic constants, V_{max} and K'. These constants indicate the "potential" for biodegradation of specific xenobiotics in natural ecosystems and thus have use in defining the overall heterotrophic biodegradation potential (HBP) of the natural microbial population present. When some background level of the substrate is present, T_n can be calculated from equation [4] by the relationship $T_n = K''/V_{max}$, where $K'' = K + S_b$ (8) and V_{max} has units of µg/liter/h.

GENERATION OF KINETIC CONSTANTS

The ability to generate HBP kinetic constants has important implications for biodegradation testing of xenobiotics. Use of these constants with other measurements of microbial biomass such as colony forming units, CFU (2), acridine-orange-direct-counts, AODC (15), and most-probable-number (MPN) determinations (7) should allow microbial numbers and degradation activity to be correlated directly via a specific activity index (SAI). The concept of SAI was initially proposed to characterize the activity of individual cells on natural substrates (13). Turnover ratee ($T_r = 1/T_n$) or V_{max} values were divided by bacterial numbers (CFU, AODC) to yield T_r or V_{max} SAI's. In principle, a similar approach should be applicable to degradation of xenobiotic compounds, if these compounds are mineralized as carbon and/or energy sources.

Figures 2, 3, and 4 illustrate the results of HBP studies on three detergent chemicals, linear alkylbenzene sulfonate (LAS), sodium nitrilotriacetate (NTA) and dodecylnonylethoxylate ($C_{12}E_9$) in ground and surface waters. All three materials were tested over a range of concentrations and rate data ($v = f/t \cdot A$) and were fit by nonlinear regression techniques to equation [6] to estimate the kinetic parameters K' and V_{max}. V_{max} was then divided by the number of viable bacteria (CFU/liter) to generate V_{max} SAI's in the different environmental samples tested.

As the data in Figures 2-4 indicate, biodegradation rate data for all three materials are accurately described by a saturation kinetic model (equation [6]). Precise estimates of the kinetic constants K' and V_{max} can be obtained (Table 1), even though maximum degradation rates vary for the detergent chemicals and natural water samples tested. Moreover, normalization of V_{max} for CFU resulted in SAI values that agreed relatively well, both within and between compound classes. This good agreement suggests that consistent kinetic results can be obtained if degradation data are normalized for both microbial numbers and microbial activity using an HBP/SAI approach.

FUTURE RESEARCH NEEDS

HBP kinetic approaches which incorporate both microbial numbers and microbial activity measurements (SAI) appear to show promise for predicting biodegradation rates of radiolabeled xenobiotics in natural ecosystems. In theory, these approaches should be applicable to die-away studies in which disappearance of parent compound is measured by specific analytical methods. More research on different xenobiotic compounds is needed, however, to establish the applicability of HBR measurements to analytical studies. In addition, several important research areas need to be addressed to determine the predictive value of an HBP/SAI approach in estimating biodegradation rates in the environment. These areas include (but are not limited to) the:

- effect of incubation period on kinetic measurements,
- significance of T_n value when $S_b \doteq 0$
- appropriate biomass measurements for generating SAI values,
- and comparability of rate data derived from short-term HBP assays vs. long-term biodegradability die-away assays.

Information is needed in these areas not only to formulate a conceptual framework for using microbial activity measurements in environmental fate research but also to establish a practical basis for predicting biodegradation rates of xenobiotics in natural ecosystems.

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Table 1. HBP kinetic constants for biodegradation of detergent chemicals in ground and surface waters.[a]

Compound	Water	V_{max} (ng/liter/h)	K' (µg/liter)	r^2	SAI (ng/cell/h)
LAS	surface	539 (429-649)	902 (546-1259)	0.99	1.5×10^{-5}
NTA	ground	138 (111-165)	63 (32-94)	0.99	1.7×10^{-5}
$C_{12}E_9$	ground	121 (105-136)	388 (256-520)	0.99	3.3×10^{-5}
	surface	8081 (4575-11587)	504 (98-1008)	0.99	3.0×10^{-5}

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[a] Values in parentheses are 95% confidence intervals of true means.

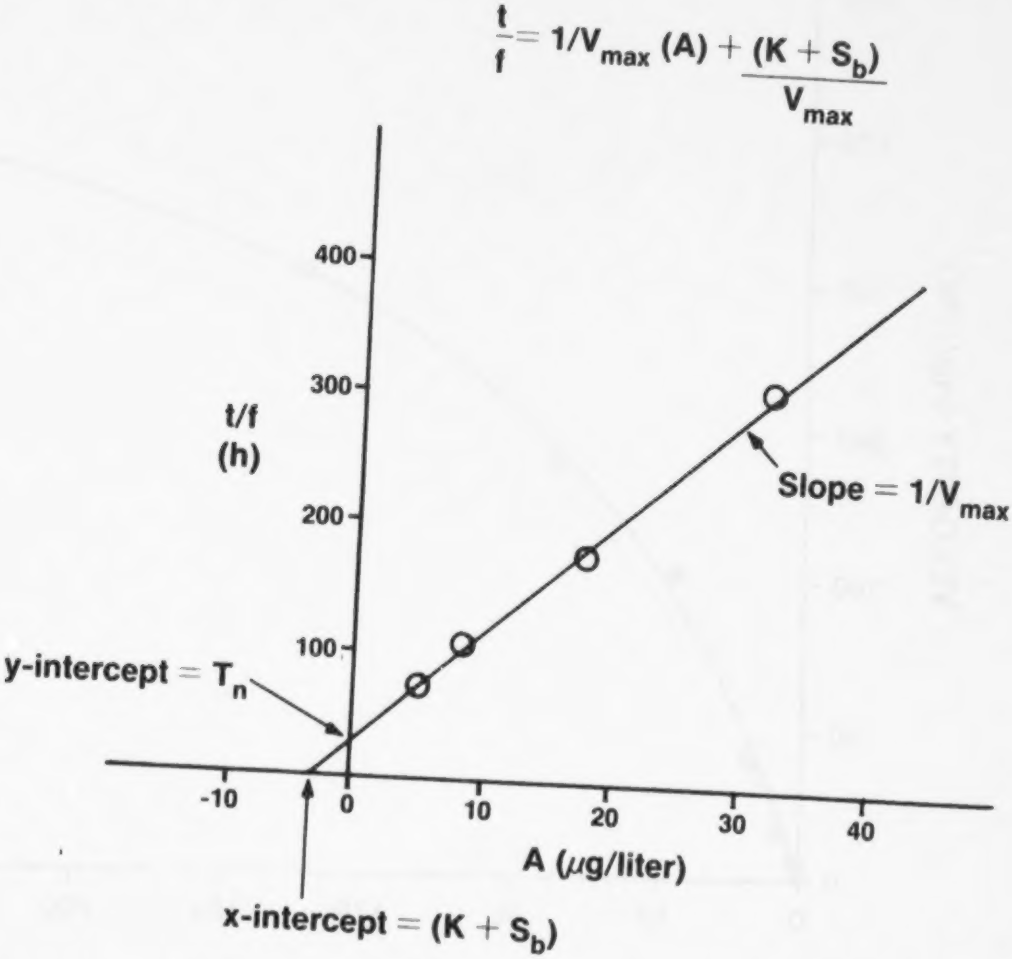

Figure 1. Linear transformation of hyperbolic saturation curve (equation (4)) as derived by Wright and Hobbie (12).

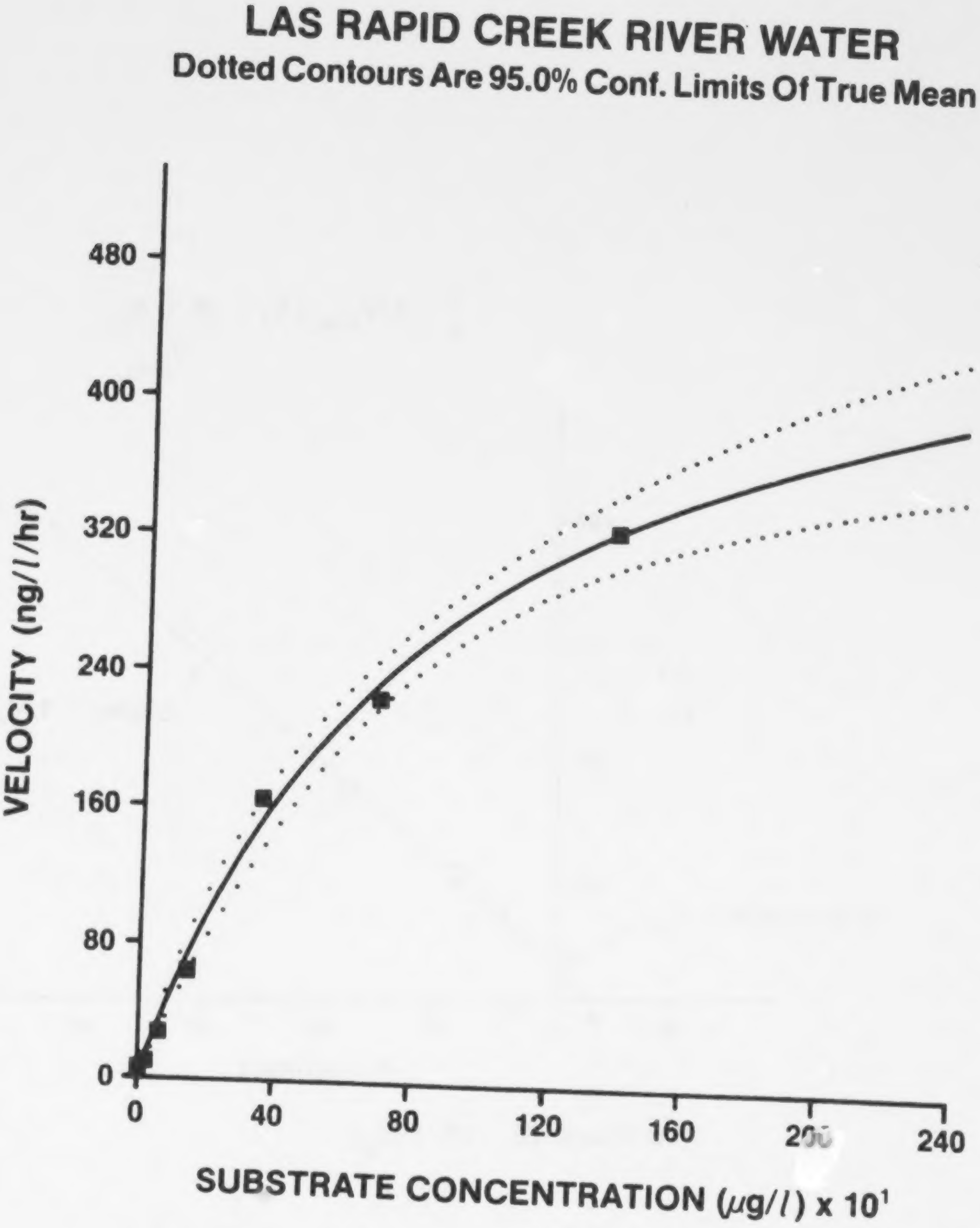

Figure 2. Rate of degradation of LAS in river water as a function of LAS concentration. Data have been analyzed by equation (6), and parameter estimates are summarized in Table 1.

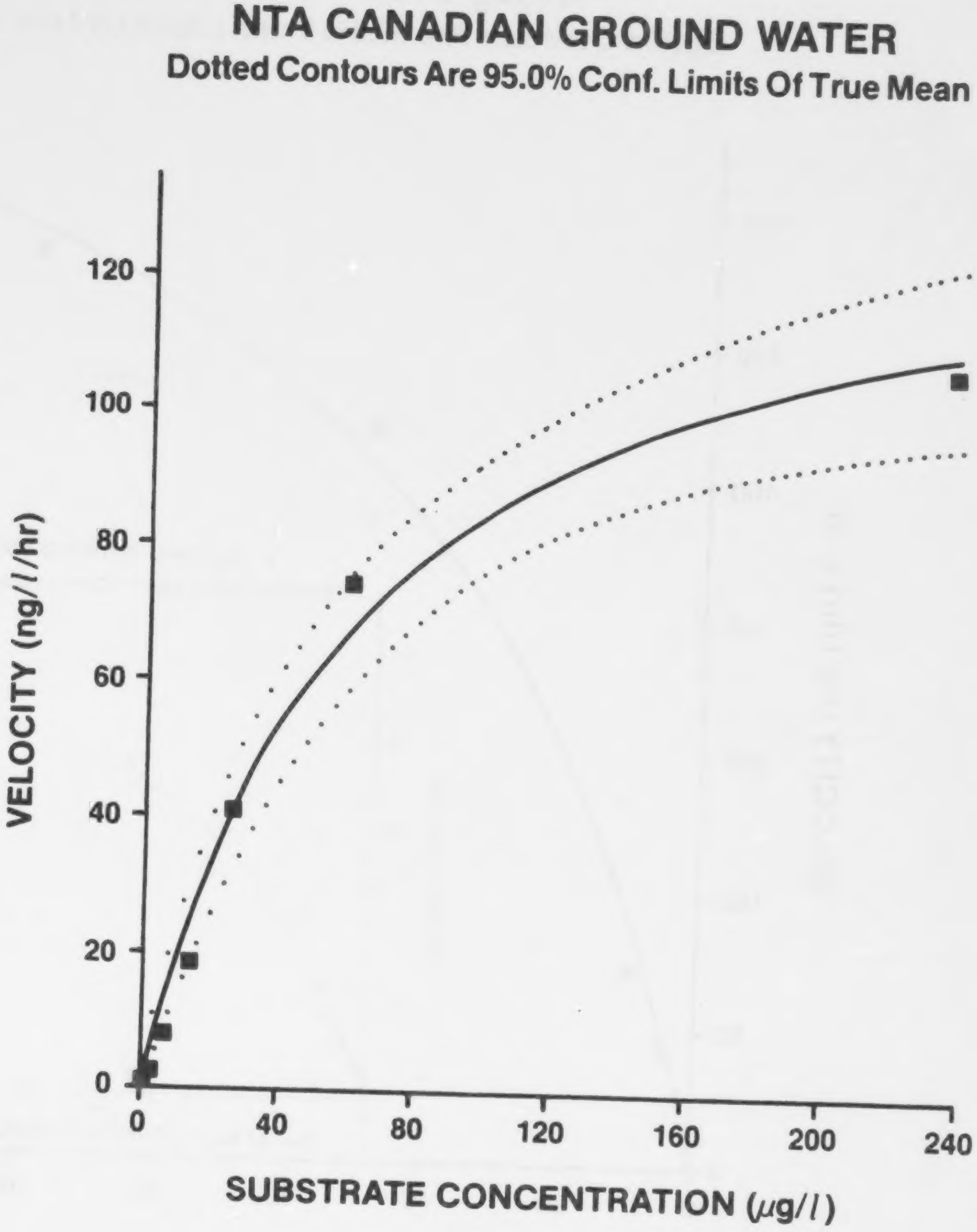

Figure 3. Rate of degradation of NTA in groundwater as a function of NTA concentration. Data have been analyzed by equation (6), and parameter estimates are summarized in Table 1.

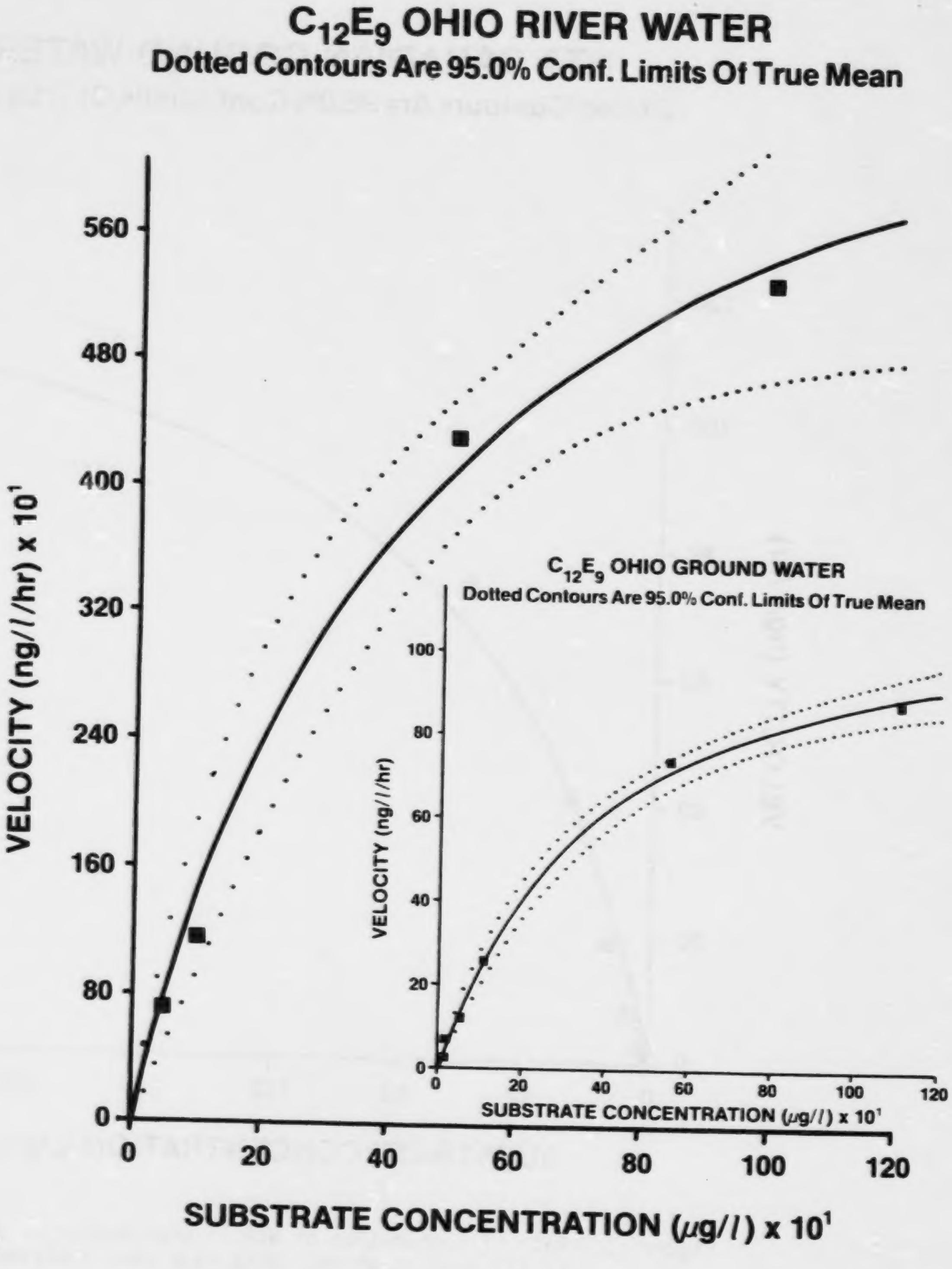

Figure 4. Rate of degradation of $C_{12}E_9$ concentration. Data have been analyzed by equation (6), and parameter estimates are summarized in Table 1.

PANEL REPORT

The purpose of this panel was to evaluate the applicability of the short-term (approximately 24 hours) heterotrophic potential method to the biodegradation of xenobiotic compounds. Before discussing the applicability of heterotrophic potential measurements, we believe it is crucial that certain terms be defined to avoid confusion and problems with semantics.

The technique commonly referred to as heterotrophic potential is an activity assessment based on saturation kinetics in which metabolic velocity is measured over a range of substrate concentrations. In addition to yielding a velocity at each concentration added, the maximum potential velocity (V_{max}) that the natural community may attain can be established. The technique is based on the assumptions specified by Michaelis-Menten which require short-term incubations to avoid significant changes in substrate concentration, active biomass, and the enzymatic rate limitating step during the testing period. Other useful biodegradation parameters can also be obtained from this data set. These parameters are defined below.

1. Specific Activity Index is a measure of the amount of microbial activity per unit biomass in which the biodegradation rate is divided by the number of organisms active on that compound. The units are rate/unit biomass.
2. V_{max} is the rate of biodegradation at saturating substrate concentrations; the units are g substrate/liter·hr.
3. Turnover time is the reciprocal of turnover rate, which is the fraction of material biodegraded at subsaturating substrate concentrations divided by time, in units of time.
4. K_t is the concentration of a compound at half V_{max} in units of grams per liter.

There are several advantages to the heterotrophic potential testing method to survey the biodegradability of xenobiotics.

1. The test is relatively simple to perform.
2. A wide range of concentrations of the compound can be tested in a relatively short period.
3. These substrate concentrations approximate realistic environmental concentrations.
4. The test is short-term (approximately 24 hours); therefore changes in certain environmental parameters (biomass, etc.) do not interfere with subsequent data analyses.
5. There are statistical techniques (nonlinear regression) available for data analyses.

6. Several useful biodegradation rate constants and kinetic constants can be calculated
7. The procedure is cost effective compared to long-term testing now performed.

As with any test method, there are also disadvantages which can restrict the uses of this method:

1. If "adaptation" by the microbial community is required, it may not be detected.
2. A radiolabeled compound is usually required to perform the tests at environmentally realistic concentrations.
3. For the test to be valid, it must be run at a range of concentrations, including levels well in excess of K_t.
4. Kinetically diverse microbial assembleges are not strictly Michaelian.

Further research is needed to assess the applicability of heterotrophic biodegradation rates in complex environmental systems. These research areas are:

1. Expansion of the data base by application of the method to other ecosystems, in particular soil and subsurface environments, by using a range of compounds (benchmark compounds) with different physical/chemical properties.
2. Definition of the range of compounds for which the method is applicable by systematic testing of different classes of chemicals.
3. Determination of the most "appropriate" measure of microbial biomass for estimating numbers of substrate utilizers and Specific Activity Indices.

Also, the panel recommends the following:

1. Data analysis should be performed by nonlinear regression techniques to estimate kinetic constants and associated statistical parameters.
2. In all testing the importance of the dilutent must be emphasized.
3. Rate constants and kinetic constants obtained from different testing protocols should be compared.
4. Future improvements in methods and theory should be incorporated into the assessment of biodegradability as technology permits.

Panel Members: Peter Chapman, University of Minnesota
Doris Paris, U.S. EPA
Gerald Neimi, University of Minnesota, Duluth
Dave Gibson, University of Texas
Bill Gledhill, Monsanto
Phil Howard, Syracuse Research Corporation

RELATIONSHIPS BETWEEN CHEMICAL STRUCTURE AND BIODEGRADATION RATES

Objective: To discuss concepts for relating the biodegradation rate of a chemical to its structure.

Because of the extremely large number of chemicals which the Office of Toxic Substances will be required to regulate, it is important to evaluate structure-activity relationships (SAR) as a potential tool for applying biodegradation information for representative chemicals to other similar chemicals for which relatively little fate information is available. Current SAR studies use BOD's and persistence testing data to compare chemical structure and chemical properties to biodegradability. These data are frequently generated using sewage sludge or other environmentally unrealistic inocula. Can biodegradation rate information from studies using natural inocula and intact microbial communities be used successfully in SAR studies? What limitations and disadvantages are inherent in this approach? What methods and quantitative analysis of the data should be used for obtaining biodegradation rate information? Is the information too site-specific? What chemical classes should be tested? What aspects of chemical structure or chemical properties should be considered?

STRUCTURE-ACTIVITY RELATIONSHIPS (SARs)

P. J. Chapman

ABSTRACT

In attempting to relate activity to structure in biodegradation studies, the object is to determine whether one can perceive in the structure of a given chemical (or in certain of its properties) features which reveal its biodegradability or its persistence. Put another way, we might ask are there ways of looking at the structures or derived parameters of organic compounds which can indicate whether their biodegradation is permitted or constrained in the environment? This approach, if successful, would of course be invaluable to the EPA Office of Toxic Substances in its evaluation of the numerous chemicals for which regulatory decisions are required.

EVALUATION OF ASSUMPTIONS

Is it possible that for kinetic purposes we can treat the complex microflora of different environments as if they are pure cultures? Or, to take the analogy one step further, as if we were dealing with a purified enzyme system challenged by a range of potential substrates? This latter analogy at least enables us to see that we can apply this information to a new, potential substrate in an informed way once we find the answers to the following questions:

(i) What is the mechanism of the reaction catalyzed?
(ii) What is the range of compounds serving as substrates and at what relative rates for given concentrations?
(iii) What groups in the substrate are recognized by the active site and what steric factors limit the range of substrates?

But can we now predict the rate of its attack? Or are we merely limited to statements such as "it looks as if it ought to fit at the active site?"

We should realize that in other areas some degree of success has already been achieved by this approach. Perhaps we can view the many different environments of our planet in the same way that a pharmacologist views tissues or target organs and asks: What is the fate of this drug in these tissues when it is administered? Pharmacologists already have at hand the knowledge about what methods of administration to use to best reach a given tissue and what types of compounds will enter these tissues and undergo metabolic transformation. In other words, the design of tissue-targeted drugs has considerable rationale. As long ago as 1690 the British philosopher, John Locke, wrote in his "Essay Concerning Human Understanding": "Did we know the mechanical affections of the particles of rhubarb, hemlock, opium and a man as a watchmaker does a watch we should be able to tell beforehand that rhubarb will purge, hemlock kill and opium make a man sleep." Note that Locke recognized the need to know both the "mechanical affectations" of man as well as those of different particles. The pharmacologists' success in relating specific structural features of drugs to precise biological effects is based on exactly those principles. Their application is relevant to an assessment of the toxicological effects of chemicals in our environment, but we should, however, realize that our level of understanding of the fate of chemicals in our environment will be limited by what we know of the potential of the environment to deal with chemicals of different types. Whether that can be treated in such a systematic fashion remains to be seen. No matter whether the assessment is toxicological or biodegradative, one is obliged to make reference to a data base -- an organized body of information to which reference can be made for precedents and examples whenever a new chemical comes under the gaze of a regulatory agency. What would our assessment now be of the early alkylbenzene sulfonate detergents in use in the late 1950's? Hopefully we would now be able to recognize that the extensive branching of the alkyl sidechains -- a feature introduced by the synthetic methods employed -- is an environmentally undesirable feature in such compounds because highly branched alkanes show limited rates of attack and the detergent sidechains are the usual sites of attack when biodegradation is initiated. Unfortunately we had to experience the effects of these slowly degradable detergents before research revealed that detergents with linear sidechains, the presently-used LAS detergents, are much more readily biodegradable.

FEASIBILITY OF SAR APPROACHES FOR BIODEGRADATION

The above example raises a question about the general feasibility of the SAR approach -- our knowledge of the biodegradation of different classes of organics has not begun to keep in step with the range of different synthetic compounds in current use, not to mention those presently under development. It would appear that at this time we do not possess a sufficiently adequate data base from which to assess different chemicals. Thus we really have at least two important questions to deal with. Is the SAR approach a feasible one? If so, then what specific information is needed to make the approach generally useful? If the answer to the first is in the affirmative, then it is necessary to evaluate different approaches perhaps by determining the predictive value of each in relation to subsequently performed laboratory and field studies with a representative number of test chemicals.

FUTURE RESEARCH NEEDS

Whatever approach is adopted, a body of information on biodegradation must be available and organized in such a way as to be useful and accessible to all interested parties. Furthermore it should be based on standardized, widely acceptable procedures. Whether we rely on a large body of existing biodegradation data such as the extensive BOD values in the literature, on pure culture studies of pathways and biotransformations or, more realistically, make use of some measure of the rate of disappearance of parent compound, are matters for discussion. Personally I favor the latter approach coupled wherever possible to some measure of the completeness of the biodegradation process using, for example, percent conversion to carbon dioxide or, in anaerobic situations, to carbon dioxide and methane. I think it should be stressed that if one adopts disappearance of parent compound as a measure of biodegradation, that an apparently rapid reaction such as, hydrolysis of the methyl ester of 24D or of methyl parathion, will yield readily degradable methanol plus the bulk of the molecule which may be considerably more resistant to attack. In other words, we have to have an approach that will also provide information about products so that these too can be evaluated in turn.

Additional questions about concentrations of test compounds, type of inoculum, whether sediment should be present or if nutrients should be added, are all reflections of the type of environment we are trying to simulate in the laboratory. Do we want to try for the best possible circumstances for biodegradation or the worst possible scenario? To me there seems to be a very real need to assess the potential for aerobic biodegradation in both types of environments and to follow up with anaerobic studies for compounds which emerge as persistent or slowly biodegradable. We could ask this question another way by saying how far can we extrapolate findings from one set of environmental conditions, inocula, concentrations, etc.

A PRELIMINARY MODEL TO PREDICT BIODEGRADABILITY FROM CHEMICAL STRUCTURE

G. J. Niemi
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Dinsh D. Vaischnav
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ABSTRACT

The long-term success in understanding the impacts of potentially hazardous chemicals in order to ensure environmental protection is dependent, in part, on the development of predictive models that reduce the need for cost-prohibitive tests on individual chemicals. Under the assumption that chemicals of similar structure have similar behavior, knowledge on the structure of a chemical allows the estimation of its activity by means of comparisons with chemicals of known activity. We present the results of a preliminary analysis on the prediction of whether a chemical is persistent or not persistent on the basis of structure-activity relationships. We use a data base derived from a literature search of the chemicals that have been tested for biochemical oxygen demand (BOD) and apply multivariate analysis to develop a prediction model.

APPROACH FOR PREDICTION OF BOD

We calculated three sets of molecular connectivity indices for those chemicals with BOD data [framework, simple or bond-corrected, and valence-corrected indices (**Kier and Hall, Molecular Connectivity in Chemistry and Drug Research, Academic Press, NY, 1976)**].

Our approach for the prediction of BOD was a two-step process: (1) identification of chemicals using similar structure with K-means clustering (**Dixon and Brown, Biomedical Computer Programs, P Series, Univ. of California Press, 1979) and (2) separate discrimination of** chemicals within a cluster of high **BOD** (defined as those with BOD values > 13) and those with low BOD (those with BOD values ≤ 13). We performed the cluster analysis with principal components that were calculated for each chemical from a separate principal component analysis of 45 variables (a combination of variables calculated from the 3 molecular connectivity indices) for 16,121 chemicals. We assumed that this large data base was representative of the multivariate space occupied by a substantial number of industrial chemicals. For example, there are currently about 45,000 chemicals registered in the Toxic Substance Control Act (TSCA) inventory.

MODEL DEVELOPMENT AND APPLICATION

A total of 340 chemicals (120 with low BOD and 220 with high BOD) **was** identified from the data base assembled by Vaischnav. Because the K-means clustering algorithm uses Euclidean distances to define clusters, chemicals that are outliers in the data base have a major influence on the definition of clusters. Therefore, we identified two major parts of the principal component space, "outer" and "inner" space, and performed separate clustering analysis on each. The outer space chemicals were defined approximately as those chemicals > 2 standard deviations from the mean for any of the first 8 principal components. This division resulted in the identification of 62 chemicals (18%) in the outer space and 278 (72%) chemicals in the inner space. Subsequently, we identified 4 clusters (clusters 1-4, Table 1) in the outer space group of chemicals and 6 clusters (clusters 5-10, Table 1) in the inner space group of chemicals.

Three of the clusters (clusters 1, 2, and 5) required no discrimination between high and low BOD groups because two of the outer space clusters included only low BOD chemicals, while one of the inner space clusters included 95% (18/19) of chemicals with high BOD. Discrimination between high and low BOD chemicals in the remaining 7 clusters was performed with stepwise discriminant function analysis (DFA) (Dixon and Brown, op. cit.). We included variables up to 6th order from each connectivity index separately in the DFA to identify those variables or combinations of variables that best separated the groups. In the summary (Table 1) we selected those variables that provided the best discrimination in terms of the number of chemicals correctly classified into the low BOD group.

The clustering/discrimination model resulted in the correct classification of 78% (92/120) of the chemicals identified as having low BOD and 77% (170/220) of the chemicals identified as having high BOD. However, there is a differential probability of correct classification based on which part of the subspace or cluster the chemical is assigned. For example, if a new chemical is classified into the outer space group of chemicals, then there is a high probability (about 94%) that the chemical can be correctly classified as having a high or low BOD. Similarly, if a chemical is classified into cluster 3 of the inner space, then there is about an 82% chance of correct classification. In contrast, the discrimination probabilities in cluster 4 or 5 of the inner space for the low BOD groups are only slightly above 50%.

FUTURE RESEARCH NEEDS

It is apparent that chemicals are persistent because of a variety of microbial mechanisms that relate to degradability. Therefore, a global approach that puts all persistent chemicals in one group and all non-persistent chemicals in another, such as a discriminant analysis of all chemicals considered simultaneously, is unsatisfactory. Our second approach was to classify chemicals into similar, relatively homogeneous groups, and then search for the common structural elements that separated persistent from non-persistent chemicals. In general, this preliminary model is relatively good for the prediction of persistence, but we believe that the model can be improved considerably. The following should be considered in subsequent applications.

1. We suspect errors in the BOD values due to a lack of acclimation of chemicals or due to test measurements. Re-evaluation and re-testing of chemicals will be necessary to clear potential problems in the data base.

2. The number of possible combinations of clusters and variables that can be used for 340 chemicals is enormous. If chemicals within a cluster have low BOD but different structural reasons for being persistent,

then there is no guarantee that discrimination analysis can recognize these structural differences. A variety of clustering models using combinations of different numbers of clusters and variables needs to be evaluated.

3. **We used the multivariate techniques described here as exploratory tools to elucidate structural configurations that may be associated with biodegradation. Therefore, we included variables with relatively low F-ratios to enter into the discrimination equation. Subsequent investigations, preferably with a larger sample size, need to be attentive to potentially spurious correlations in the process of screening a large number of variable for inclusion in a model. For this reason we have emphasized the potential usefulness of this overall strategy and have not focused on specific structural attributes that were associated with discrimination.**

Table 1. Summary of cluster analysis and discriminant function analysis for 340 industrial chemicals.

SPACE	CLUSTER ANALYSIS			DISCRIMINATION ANALYSIS		
		N-Chemicals		% Correctly Classified		
		High BOD	Low BOD	High BOD	Low BOD	Variables Included -F Ratio
Outer	1	0	6	-	-	-
	2	0	16	-	-	-
	3	5	13	60	85	SCL4 - 4.95 SCL6 - 2.03
	4	13	9	85	78	FCL5 - 4.92 FP6 - 5.40 FP4 - 2.84 FCL3 - 1.28
Inner	5	18	1	-	-	-
	6	35	18	60	78	SPC6 - 1.18 SPC5 - 2.67
	7	10	12	80	83	VPO - 1.16 VP1 - 2.23 VCL4 - 3.83 VCH6 - 2.27
	8	60	15	83	53	VPO - 4.78 VP3 - 7.60 VP4 - 3.04 VCL3 - 1.37
	9	29	14	72	64	SCL5 - 2.63 SCH5 - 3.79 SPC4 - 2.55 SPC5 - 1.65
	10	50	16	76	75	SP1 - 2.63 SP3 - 4.12 SP5 - 1.42 SCH6 - 5.91
Total		220	120	77	78	

PANEL REPORT

To obtain information on the biodegradation of representative compounds for a data base by which to assess the biodegradation of many other similar chemicals of interest to OTS, we employed two general approaches. The first of these makes use of pure cultures, an approach that reveals only the genetic potential of microorganisms to bring about biodegradation. It does not supply information about the role of these organisms under environmentally realistic conditions. The second approach employs mixed cultures under conditions that simulate environmental circumstances and provides biodegradation information such as the kinetics of compound disappearance and the need for acclimation periods. It should be stressed that the only test systems from which reliable data extrapolation can occur are those that employ mixed cultures and environmentally realistic conditions.

In the view of this panel, it does not appear to be possible to predict biodegradation rates from structure-activity relationships (SAR). For a small category of molecules, however, it does appear possible to make qualitative or even semi-quantitative judgments. The limitation is due to lack of a sufficient data base. Compounds which are identifiable as the simpler members of discrete chemical classes, such as aromatic hydrocarbons, esters, phenols, and amides, generally fall into this category.

If we are to explore whether predictions of a qualitative or perhaps at best a semi-quantitative nature are possible, it is necessary to enlarge and extend the base of information from which extrapolations might be made. In this connection it is important that an acceptable procedure is adopted by a majority of workers in this area. Use of BOD data has suggested that SAR correlations can be profitably pursued but it appears that a more acceptable methodology should provide a reliable measure of primary biodegradation under conditions simulating different environments and with regard to ranges of substrate concentration variations such as temperature, aerobic/anaerobic conditions, presence of sediment, eutrophic versus oligotrophic water, etc. This panel recommends that such a procedure be adopted for the purposes of generating information useful for SAR considerations but wherever practicable should be accompanied by parallel studies of rate measurements of ultimate biodegradation.

Having generated a body of primary biodegradation data for various classes of chemicals, it should be evident what chemical or physical parameters might logically be explored for correlations that might provide indicators of biodegradation. Chemicals should be selected so that they can be grouped into different clusters based on chemical classes by functional groups. Within these clusters applications of statistical techniques can establish whether significant correlation exists between structure and biodegradation rates.

We suggest that the following are workable approaches which should be implemented into research plans:

1. Choose optimal test systems acceptable for wide-spread use.
2. Choose chemicals or classes of chemicals from a broad cross-section of those representative of different classes of environmental interest.

3. Determine whether biodegradation data for simple compounds can be related to more complex structures possessing several of the same structural entities.

Panel Members: Gary Klecka, Dow Chemical
Dick Raymond, Groundwater Environmental Consultants
Jim Spain, Georgia State University
Harvey Holm, U.S. EPA
John Walker, U.S. EPA
Robert Brink, U.S EPA
Art Kaplan, U.S. Army-Natick

EXTRAPOLATION OF LABORATORY BIODEGRADATION DATA TO THE FIELD

Objective: To develop a conceptual and experimental framework for testing the usefulness of kinetic expressions of biodegradation processes in predicting that biotic fate of chemicals in complex natural situations.

Decay curves are commonly obtained from laboratory experiments in which little attempt is made to maintain conditions similar to those in the field. Resulting biodegradation rates may, therefore, be potentially inaccurate estimations of rates in the field. What should be done to test the environmental significance of these rate determinations? Are field studies required? How should field studies be designed? What is the minimum size and scope for a field test? What additional environmental factors should be examined for their effect on biodegradation? How will laboratory-derived rates and their associated kinetic expressions be used to predict biodegradation in the field where the effect of variations in environmental factors may be additive or integrative? What is the role of mathematical models in relating laboratory data to the field?

EXTRAPOLATION OF LABORATORY BIODEGRADATION DATA TO THE FIELD

Gary M. Klecka

ABSTRACT

Studies on the biodegradation of chemicals in the environment are extremely complex. In addition to microbial transformation, other environmental processes such as volatilization, photodecomposition, hydrolysis, and adsorption are likely to influence the concentration of a particular chemical. As a result, model systems are usually established in the laboratory to study the decomposition of a compound under controlled conditions, and the rates observed are then extrapolated to describe the process in the field. Since the key to successful extrapolation ultimately depends on the laboratory data, special attention must be paid when designing and conducting experiments such that the information obtained will enable one to make a reliable estimate of the rates at which a process will occur in the environment.

FACTORS CONTROLLING THE FATE OF CHEMICALS IN THE ENVIRONMENT

Fundamental to predicting the biodegradation of a chemical in the environment is the identification of the environmental compartments in which the chemical is likely to reside. To address this question, a number of simple equilibrium models have been developed, such as the one recently described by Neely and Mackay (6). Once the partitioning of the compound is known, the importance of biodegradation in controlling the fate of the chemical in the environment will require an understanding of: (a) the mechanism(s) involved in decomposition, (b) the rates at which these processes occur, and (c) factors which are likely to influence the rates.

Laboratory studies with pure cultures of microorganisms and individual compounds have revealed considerable information regarding the mechanisms involved in the biodegradation of a wide variety of organic chemicals. Many of these reactions can be classified as oxidation, reduction, hydrolysis, or conjugation. One of the most important features of microbial degradation is the ability of aerobic microorganisms to catalyze the incorporation of molecular oxygen into the substrate (4). Thus, the bacterial degradation of many aromatic molecules has been shown to require molecular oxygen for both hydroxylation and ring-fission reactions. Alternatively, a number of compounds, including several of the organochlorine insecticides, have been shown to be degraded more rapidly under anaerobic conditions (7). Thus, a knowledge of the mechanisms involved in microbial metabolism provides an indispensable guide for understanding the fate of organic chemicals in the environment.

In order to define the role of biodegradation in determining the environmental concentration of a particular chemical, a quantitative expression describing the rate of the reaction is required. The microbial degradation of a number of chemicals in natural waters and soils has been shown to be described by first-order kinetics, and thus the rates of these biological reactions can be expressed in terms of rate constants. Since first-order rate constants are often subject to considerable variability, the extrapolation of rate constants to different systems will require an understanding of the importance of microbial numbers or activities in determining the reaction rate. One approach to this problem involves normalizing rate constants by the size of the total microbial population as enumerated by conventional techniques (2). However, a considerable number of studies have shown that biodegradation rates do not consistently correlate with any measurement of the total microbial population (1, 3, 10). Thus, further studies are necessary to determine the relationship between reaction rate constants and the concentration or activity of the specific microorganisms responsible for degrading a compound.

The extrapolation of laboratory-derived rate constants to the environment will also require an understanding of the various biological, chemical, and physical factors that influence biodegradation rates, and the integration of these effects into mathematical models. Biological variables include concentration, spatial distribution, species composition, activity, and previous history of the microbial population. In addition, biodegradation may be affected by interactions among members of the community such as mutualism, commensalism, competition and predation, although little is known about the influence of microbial interactions on degradation rates. Substrate concentration may also be a significant factor affecting the susceptibility of an organic compound to microbial attack. There is some evidence suggesting the existence of threshold concentrations below which biodegradation does not occur; however, the data are inconclusive. The biodegradation of organic chemicals is known to be affected by a number of environmental variables, including sorption, mixing, temperature, oxygen concentration, redox potential, pH, ionic strength, and the presence of organic and inorganic nutrients. Unfortunately, the significance of many of these variables is poorly understood, and few quantitative relationships are available. These effects will need to be evaluated if laboratory data are to be successfully extrapolated to predict the fate of chemicals in the environment.

USE OF LABORATORY DATA TO PREDICT FATE IN THE FIELD

In spite of the many uncertainties, several investigators have had considerable success in using laboratory biodegradation data to predict the fate of a chemical in the environment. The key to these initial attempts involved the application of site-specific rate constants. This approach was used by Games (5) for modeling the fate of linear alkylbenzene sulfonate (LAS) in Rapid Creek, South Dakota. LAS is introduced into the stream from a single point-source input of domestic sewage. The choice of LAS as the test substrate simplified the modeling process, since biodegradation has been identified as the principal transformation mechanism. Rate constants were measured using water and water-sediment mixtures obtained from sites downstream from the Rapid City wastewater treatment plant. The U.S. Environmental Protection Agency Exposure Analysis Modeling System (EXAMS) was then used for predicting the fate of LAS in Rapid Creek, and the results were compared to actual LAS concentrations in the stream below the treatment plant. Steady-state concentrations of LAS in the water and sediment predicted using the EXAMS model agreed fairly well with the measured concentrations. However, a sensitivity analysis of the model to errors in flow rate, aqueous and sediment biodegradation rate constants, dispersion coefficient and adsorption coefficient revealed that the model calculations were highly dependent on the least understood parameters, namely the dispersion coefficient and the sediment biodegradation rate constant. Variation in either of these parameters resulted in a significant deviation of the predicted concentrations from the measured values. It is clear that a better understanding of sediment biodegradation and sediment-water interface dynamics is required to improve predictions of the fate of chemicals in the aquatic environment.

Walker (8) demonstrated the use of site-specific rate constants for predicting the fate of the herbicide propyzamide in experimental field plots. Laboratory studies conducted using soil samples obtained from the test site indicated that biodegradation was first-order with respect to propyzamide concentration, and the effects of temperature and soil moisture on the reaction rate could be described using simple equations. Field studies were conducted on two separate occasions, and test sites were treated to simulate both surface applied and incorporated herbicide formulations. Measurements of propyzamide concentration, soil temperature, and moisture content were performed throughout the field experiment. For the computer simulations, biodegradation rate constants were corrected using data obtained for variations in soil temperature and moisture, and changes in herbicide concentrations were predicted by integration of the decomposition rates calculated for the entire experimental period. The decomposition of propyzamide predicted using the computer model was found to correlate well with the measured concentrations of both surface applied and incorporated formulations.

Walker (8) subsequently modified the basic simulation model to include methods for estimating soil temperatures and moisture contents from standard meteorological data. When used in conjunction with site-specific degradation rate constants, the model has been shown to predict the environmental fate of the pesticides simazine, atrazine, propyzamide, linuron, metamitron, trifluralin, metribuzin, chlorthal-dimethyl, prometryne, asulam, and napropamide (9). With certain compounds, such as simazine, atrazine, propyzamide, and metribuzin, there was a tendency to underestimate the rates of disappearance. With these pesticides, it appears that mobility of the compound in the soil was responsible for the inconsistencies between predicted and measured concentrations. Deviations were also noted in an attempt to model the fate of surface applied formulations of napropamide, where loss of the compound was affected by photodecomposition. These observations emphasize the importance of accounting for all fate processes when attempting to model the fate of chemicals in the environment.

FUTURE RESEARCH NEEDS

In summary, predicting the role of biodegradation in determining the fate of chemicals in the environment in many cases will involve the extrapolation of laboratory data to the field. It is clear that the key to successful predictions will require a fundamental understanding of:

1. mechanisms involved in decomposition
2. kinetics of biological reactions
3. relationship of microbial activity to reaction rates
4. environmental variables likely to influence the rates.

Although a considerable amount of research remains before reliable extrapolations of laboratory data can be made on a routine basis, several investigators have had considerable success by applying simple models in conjunction with site-specific rate constants. It is likely that further studies of this type will be important for improving our ability to predict the fate of chemicals in the environment.

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EXTRAPOLATION OF LABORATORY BIODEGRADATION DATA TO THE FIELD

Richard L. Raymond

ABSTRACT

Field experience utilizing biodegradation as a cleanup tool for hydrocarbon contaminated soils and groundwaters has shown that many factors, presently beyond the control of the operator, affect the kinetics of degradation. It seems highly unlikely that any laboratory test can be devised that will come close to describing the kinetics to be expected in the field. A few examples will illustrate the problem.

USE OF LABORATORY DATA FOR FATE PREDICTIONS IN THE FIELD

Numerous laboratory and pilot plant studies have shown that many pure hydrocarbons and mixtures thereof are degradable at rates above 0.5 lbs/gal/day. The conditions for this high rate of turnover optimize oxygen demand, substrate and inorganic nutrient concentrations, temperature, and physical mixing. Optimization is rarely achievable in the field. In Table 1, Table 2, and Figure 1, the effect of substrate type and concentration, soil composition, and probably adsorption on the rate of biodegradation is illustrated for two biofarming projects.

Equally dramatic effects are shown in field situations for groundwater cleanup of gasoline. Two examples are shown in Figures 2 and 3. Rates in gallons of aquifer storage capacity are 0.003 and 0.005 lb/gal/month, respectively. In groundwater cleanup, oxygen transfer is the most limiting factor in the rate of degradation.

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Table 1. Biodegradation rates observed in landfarming.

Substrate	Loading lbs/cu. ft.	Rate lbs/cu. ft./month
Decane	0.012	0.0061
Octadecane	0.01	0.0070
Tetramethylpentadecane	0.01	0.0020
Biphenyl	0.01	0.0070
Phenanthrene	0.02	0.0100
Phenylundecane	0.01	0.0200
#2 Fuel Oil	1.6	0.1100
#6 Fuel Oil	1.6	0.0750
Crude Oil	1.6	0.0810

These rates are orders of magnitude less than predicted from laboratory data.

Table 2. Percentage reduction in oil concentration

Type of Oil	Location of Field Sites			Average
	Marcus Hook, Pennsylvania	Tulsa, Oklahoma	Corpus Christi, Texas	
After one year:				
Used crankcase oils	69.2	73.8	60.8	67.9
Crude oils	54.2	77.5	54.2	61.9
Home heating oils (#2)	86.0	90.0	86.0	87.3
Residual oil (#6)	48.5	65.5	59.4	57.8
After three years:				
Used crankcase oils	90.6	99^+	79.8	89.8
Crude oils	87.3	99^+	78.0	88.1
Home heating oil (#2)	98.3	99^+	94.0	97.1
Residual oil (#6)	89.2	94.0	85.5	89.5

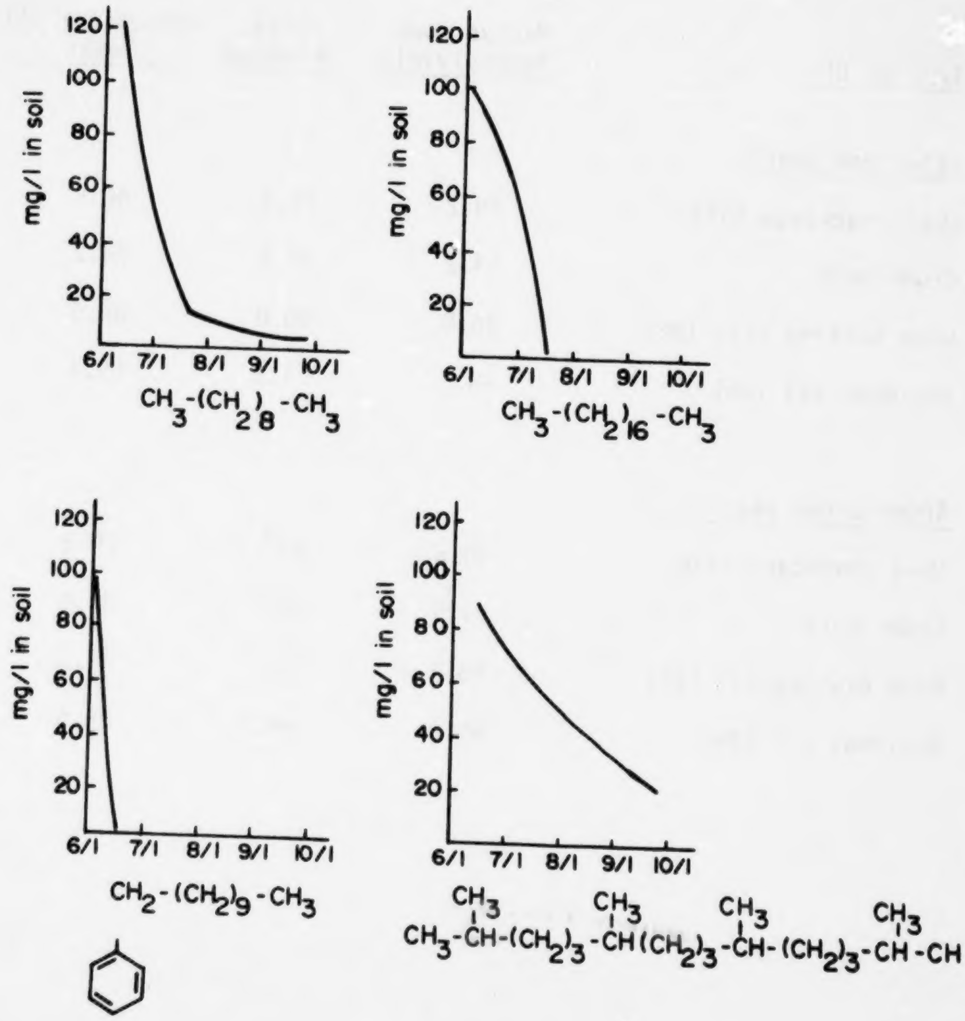

Figure 1. Examples of biodegradation under field conditions.

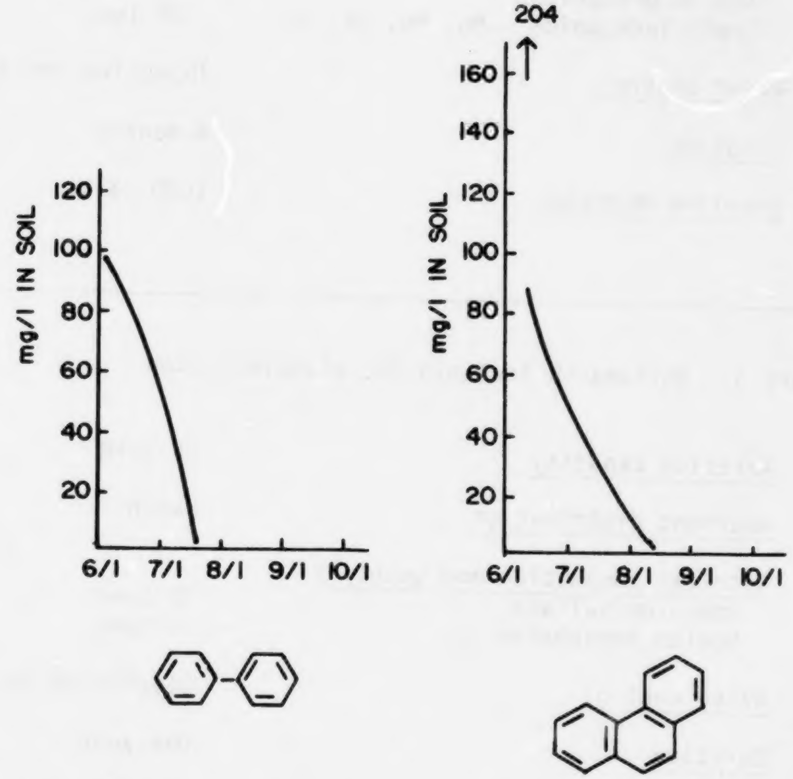

Figure 1 (continued). Examples of biodegradation under field conditions.

Figure 2. Millville, NJ, biostimulation

Aeration capacity	20 SCFM
Nutrient distribution	Batch and continuous
Nutrient compostion and quantity	
Ammonium sulfate	1200 lbs.
Sodium phosphates	600 lbs.
Trace inorganics - Mg, Mn, Fe, etc.	25 lbs.
Water control	Injection and discharge to waste
Duration	6 months
Gasoline degraded	1000 gals.

Figure 3. Whitemarsh Township PA, biostimulation

Aeration capacity	25 SCFM
Nutrient distribution	Batch
Nutrient compostion and quantity	
Ammonium sulfate	58 tons
Sodium phosphates	29 tons
Water control	Discharged to waste
Duration	One year
Gasoline degraded	45,360 gals.

COMPARISON OF p-NITROPHENOL BIODEGRADATION IN FIELD AND LABORATORY TEST SYSTEMS

J. C. Spain

INTRODUCTION

Laboratory test systems provide the most practical means to obtain data that can be used to predict the biodegradation and fate of organic pollutants. More accurate predictions could probably be made if fate tests could be conducted in the field for each chemical under consideration. Such studies have often been conducted for the application of pesticides to soil, but constraints of time and expense do not permit such studies to be carried out routinely in aquatic habitats. The second best approach is to conduct a few field tests with selected chemicals and to compare the results with those from laboratory tests to assess the relevance and utility of the laboratory data. When the strengths and weaknesses of the test systems are understood, the degree of confidence with which laboratory data can be extrapolated can be evaluated.

We have used several types of biodegradation test systems, including shake flasks, eco-cores, and microcosms, to study the biodegradation of p-nitrophenol (PNP) in the laboratory. In most instances, microbial communities degraded nitrophenol after a lag period of several days. The length of the lag period was variable, however; and in samples from estuarine or marine sites and some freshwater sites, there was no biodegradation for weeks. The inclusion of sediment also seemed to affect the biodegradation, but it was not clear whether the effect made the results more or less realistic.

The purpose of this study was to compare biodegradation of p-nitrophenol and concomitant responses of microbial communities in laboratory test systems with those in the field. We prepared laboratory test systems with samples from a freshwater pond and then treated all of the laboratory systems and the pond simultaneously with the test compound so that direct comparisons could be made. Two questions were of primary importance: (1) Is the adaptation process in the field similar to that in the laboratory? (2) Which type of laboratory test system (what level of complexity) best predicts the field results?

RESULTS FROM FIELD STUDY

In the pond, PNP disappeared slowly for the first 120-150 hours (Figure 1), then much more rapidly. Because of fluctuations in the pond water level due to rain and runoff, it was not clear from the shape of the curve between 100 and 200 hours whether the biodegradation rate had increased or whether the test compound was being washed out of the pond. Therefore, when the PNP concentration fell below 50 µg/l in the pond, an additional aliquot of

PNP was added to bring the concentration to 134 µg/l. After 23 hours with no measurable rain or runoff, the nitro compound was no longer detectable, which indicated that the biodegradation rate had increased dramatically.

The effects of prior exposure on the microbial community were determined by measuring the biodegradation rate of PNP in the pond exposed to the nitro compound and in the control which had no prior exposure. The test compound disappeared from the exposed pond within one day (Figure 2). During the same period, there was no detectable degradation in the control pond. The rate of biodegradation in the first 24 hours was two orders of magnitude faster in the exposed pond than in the control (-9.84 compared to 0.0998 µg/l). After an acclimation period of 3-4 days, the test compound was also degraded in the control pond (data not shown).

Bacteria able to mineralize PNP were enumerated in samples taken from the pond during the initial exposure to determine whether changes that would account for the increased degradation rates could be detected in the microbial community. There was an increase of three orders of magnitude in the number of PNP degraders in the exposed pond between 100 and 240 hours (Figure 3), which corresponded to the time period during which PNP degradation increased dramatically. There was no commensurate increase in total heterotrophs, and there was no proliferation of PNP degraders in the control pond.

RESULTS FROM LABORATORY TESTS

Results in laboratory test systems were similar to those in the pond. For example, data from the small microcosms and from shake flasks with sediment are shown in Figures 4 and 5, respectively. The shake flasks without sediment (Figure 6) were the only exception to the close correlation between laboratory and field data. The lag period extended for almost 500 hours, and the subsequent degradation rate was slower than in the other systems.

Increases in biodegradation rates correlated well with increases in populations of PNP degrading bacteria. We are currently investigating the relationships among microbial growth, acclimation, and degradation of xenobiotic compounds.

The results of this study show that there can be dramatic increases in microbial degradation rates in the field after exposure of the resident microbial community to the test compound. Such increases must be considered in predicting the fate of pollutants that could serve as growth substrates for bacteria. Our earlier work has shown that there can be wide variations in the abilities of communities to adapt to degrading a test compound. We now have a better understanding of how laboratory test systems can be used to assess the biodegradation responses. For example, the role of the sediment in the acclimation and the degradation is readily apparent. We are currently conducting experiments to determine the factors that control such responses so that they can be predicted.

CONCLUSIONS

Several conclusions can be drawn regarding extrapolation of laboratory data to predict results in the field:

1. Laboratory systems must include the components that affect the biodegradation rates and the complexity necessary to simulate the environment under consideration. In this case, inclusion of sediment from the test site provided an acceptable level of agreement.

2. Measurement of biomass or any other surrogate for predicting biodegradation probably will not provide predictions that are as accurate as measuring the biodegradation rate in the environment.

3. If a simple biodegradation test system can be shown to predict the biodegradation process in the environment, the most reasonable approach would be to use the test system with an inoculum from the site to predict biodegradation at that site. That is to say, if some parameter must be measured to predict biodegradation rates at a particular site, then measuring the biodegradation rate itself with a simple laboratory system would be the most effective means currently available.

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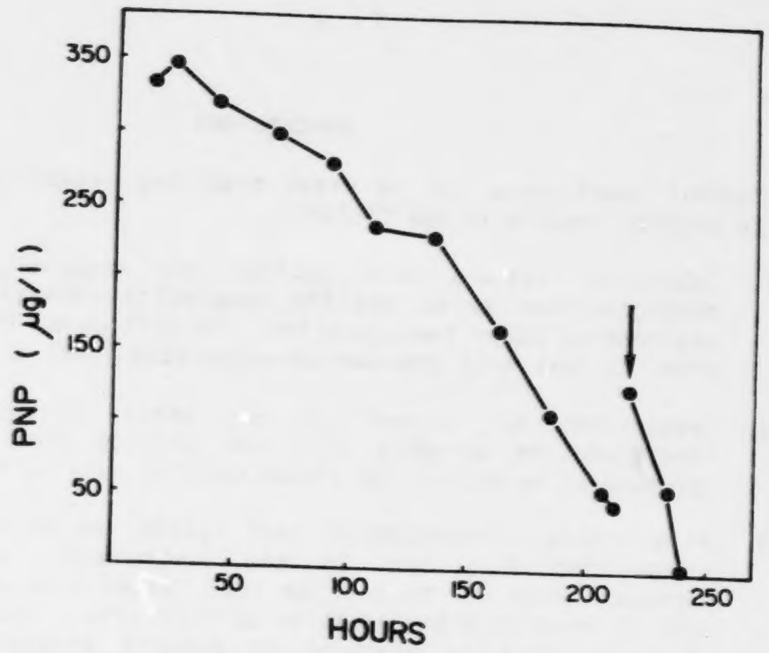

Figure 1. Concentration of PNP in the test pond. The test pond received an additional treatment of PNP after 220 hours.

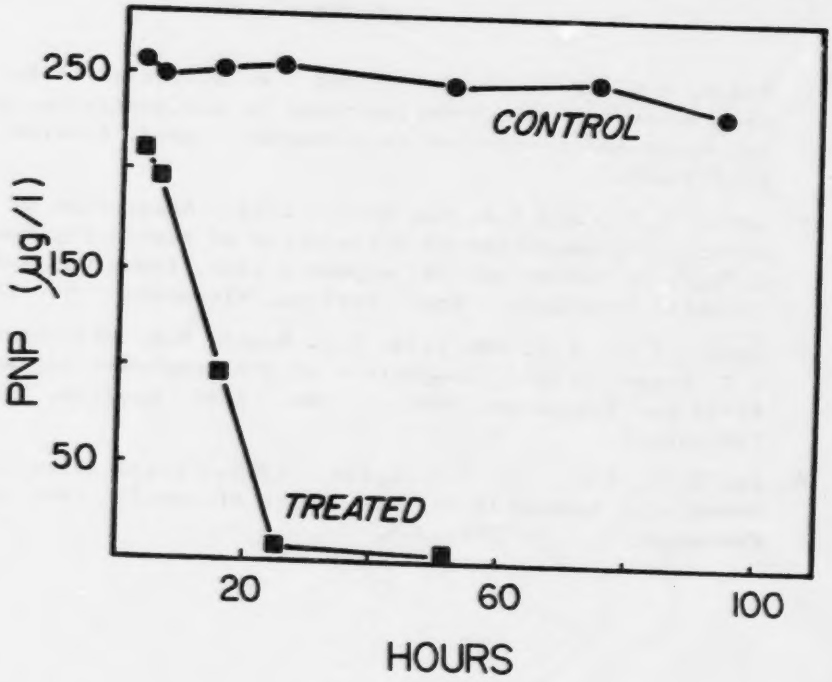

Figure 2. Concentration of PNP in treated and control ponds. The treated pond was exposed to PNP for 10 days; the control pond received no pretreatment.

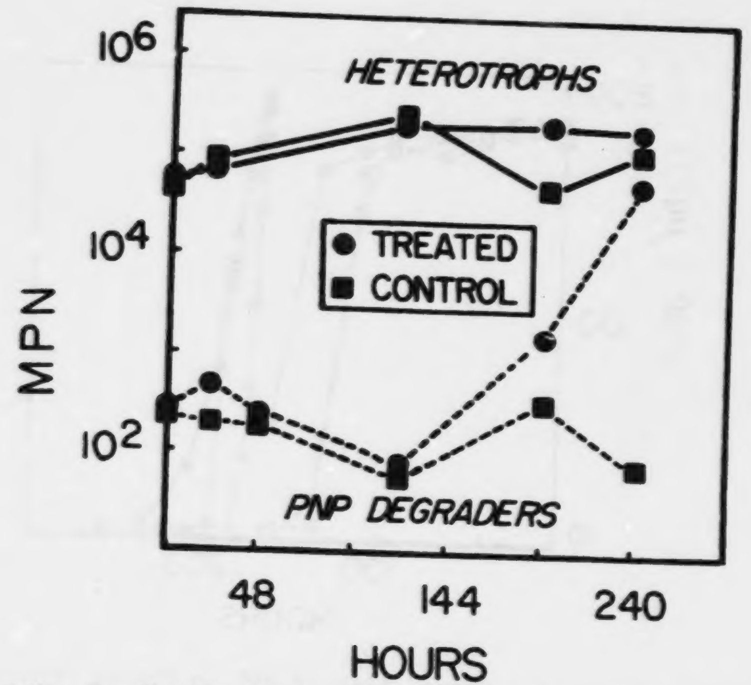

Figure 3. Total heterotrophs and PNP degraders enumerated by MPN technique in previously treated and control ponds. Samples were diluted appropriately and incubated in PNP nutrient media for 2 weeks.

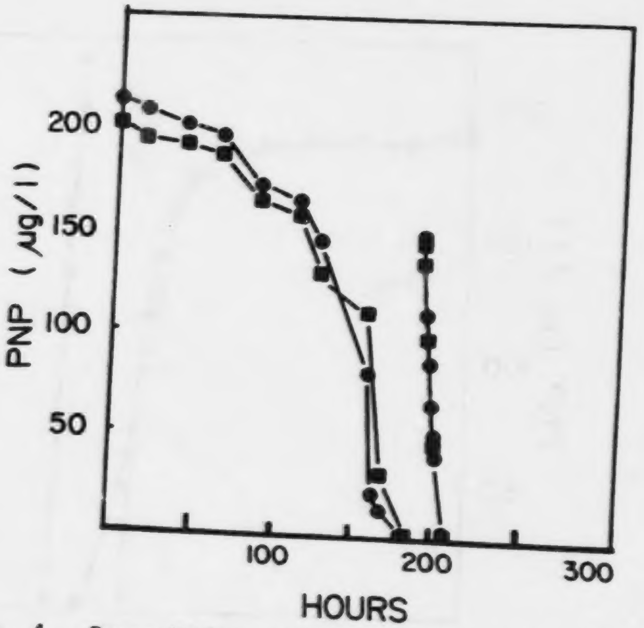

Figure 4. Degradation of PNP in small (3 l) microcosms. The microcosms contained sediment and water (removed prior to treatment from the pond). The microcosms received an additional treatment of PNP after 180 hours.

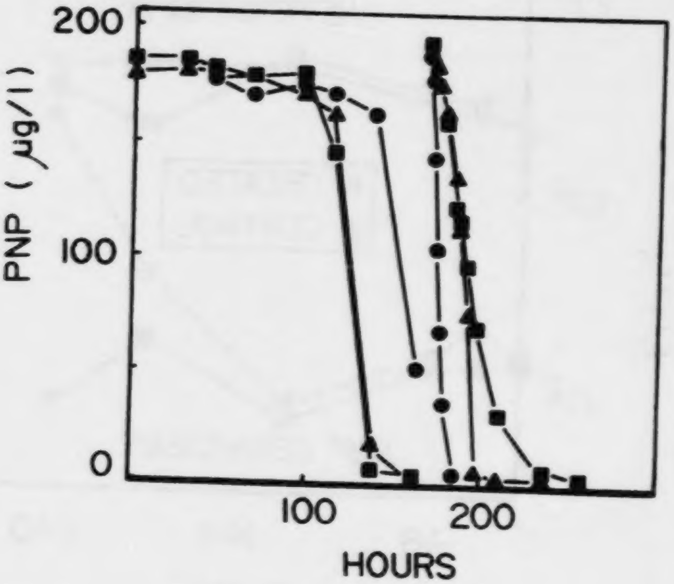

Figure 5. Degradation of PNP in flasks containing water and 500 mg sediment/l. The sediment was removed from the pond prior to treatment. The flasks received an additional treatment of PNP after 170 hours.

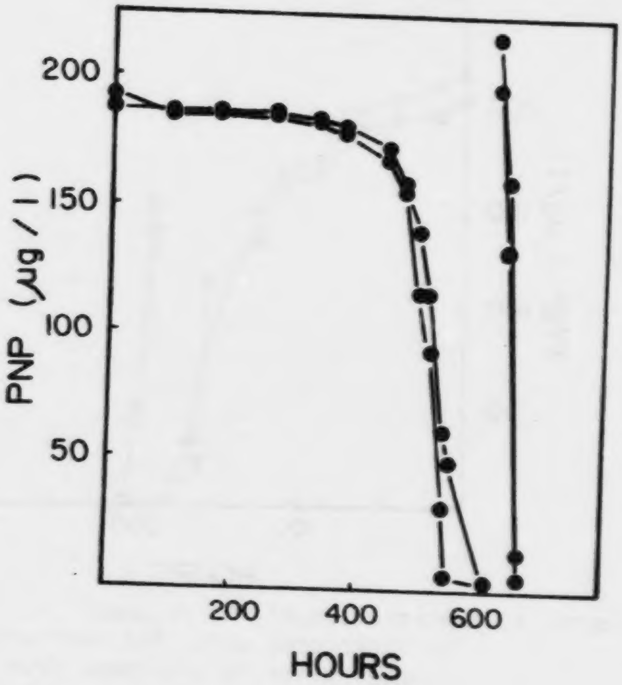

Figure 6. Degradation of PNP in flasks containing water from the field site. The flasks received an additional treatment of PNP after 600 hours.

PANEL REPORT

The importance of field studies has become increasingly evident to participants at this workshop. At this point, there is considerable uncertainty among workshop participants regarding the validity for extrapolating laboratory data to predict the biodegradation of a chemical in the environment. Consequently, the validity of laboratory studies must be tested in the field.

Fundamental to this process is the selection of laboratory test systems that are similar to the various environmental compartments (soil, water, groundwater, etc.). Each of these laboratory systems should incorporate the important components of the compartment under consideration, such as a field source of inoculum, the appropriate solid surfaces (e.g., sediment), and environmental conditions. Once a laboratory system is developed, it will then be important to verify the ability of the system to predict the biodegradation of a chemical in the corresponding field site. The design of these experiments should incorporate a statistical analysis, in order to minimize the variability likely to be encountered in the environment. Should the results of initial trials indicate that the laboratory system does not predict the field observations, the system should be modified as appropriate.

Upon establishment of a reliable laboratory model, the system should be validated by using several chemicals of different classes and enough field sites to establish confidence in the system. Following validation, the laboratory system can then be used as a tool to predict the biodegradation of other chemicals at a given site. Furthermore, the laboratory system can also be used with confidence to address a variety of additional questions, such as the relationship of biodegradation rates among different sites and the effects of a controlling environmental factor on the biodegradation rate.

SUMMARY AND RESEARCH PLAN FOR BIODEGRADATION STUDIES

BASED ON OUTPUTS AND DISCUSSIONS AT THE BIODEGRADATION KINETICS WORKSHOP

J. M. Suflita
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INTRODUCTION

The microbial ecology effort of the EPA has evolved to the point where it is no longer sufficient for researchers to enumerate the numbers and types of microorganisms in a particular environment. Past experience has taught us that this approach provides little insight into the cycling of materials and energy in the ecosystem to which the organisms belong. More importantly we have become interested in and involved in the activity of microorganisms in nature and wish to describe that activity in a quantitative manner. Therefore, we feel that future research should describe kinetics of microbial activity, predominant variables controlling the reaction rates, and potential techniques to extrapolate laboratory biodegradation information to the field.

The greater degree of complexity and sophistication inherent in this research endeavor presents numerous problems in approach and methodology. The biodegradation kinetics work has pointed up a number of equivocal approaches and interpretational difficulties in biodegradation studies. However, during the course of this meeting, certain underlying generalizations were established; these can serve as a basis for the formulating of future research directives.

We have attempted to organize these directives into a specific research plan. Clearly, the various avenues of kinetic research are not mutually exclusive. Progress in one area will assist the development of another. However, we feel strongly that certain research areas deserve an immediate effort, whereas others will evolve in the future.

Kinetic models of biodegradation processes were generally considered by the participants at the workshop as necessary elements of a research program. Models are based on assumptions that can form the basis for specific testing of hypothesis and related experimentation. They force us, for pragmatic reasons, to describe the complexity of biodegradation with a minimum number of measurable parameters. The parameters idealy represent an integration of the most important biotic and abiotic factors affecting the biodegradation of xenobiotic chemicals. Although the time and research needed to search for and test those parameters will be extensive, we feel that the potential outcome will justify the effort. Kinetic models will also provide predictive capabilities that will be invaluable for our making regulatory decisions. Such models will require us to examine the factors controlling adaptation events in natural ecosystems, since these phenomena must be included in our predictions. We believe that the testing of kinetic models represents a major research directive.

Equally important are research endeavors focusing on the problem of extrapolating laboratory biodegradation data to the field and extending biodegradation kinetic predictions from one ecosystem to another. The development of a conceptual framework for extrapolation will be the impetus and direction for gathering data from both field and laboratory experiments. It will also force us to make decisions about how to determine efficiently the spatial and temporal variability in biodegradation rates. In addition, it will facilitate the regulatory decision-making process and base it on observed variability.

These two areas, model testing and extrapolation, will therefore form the basis for our research plan. Discussions and panel reports from the workshop helped define the goals of this plan.

MODEL TESTING

KINETIC MODELS FOR BIODEGRADATION

Several types of kinetic models of varying degrees of complexity were discussed at the workshop. However, two simple models, a first-order decay model and the so-called "second-order approach" or Athens model, as we will subsequently refer to it, seem to be the most reasonable to incorporate into a research plan.

A first-order decay model contends that the rate of substrate (xenobiotic chemical) disappearance over time in any environmental sample will depend on the substrate concentration by a proportionality constant (k_1) if other conditions in the test system (cell numbers, cell activity, nutrients, competing substrates, etc.) remain the same over the course of an experiment. The proportionality constant is a reflection of the actively degradating microbial biomass in the sample being tested and the inherent rate at which the enzymatic machinery in the cells will attack the xenobiotic chemical in question. It obviously will vary, depending on when and where the environmental sample is taken. The model's dependency on substrate concentration will only be true when substrate concentrations are relatively low.

The Athens model reasonably contends that the rate of biodegradation is proportional to both substrate concentration and actively degrading biomass. Typically, a first-order rate constant (k_1) is normalized by a biomass measurement to produce a proportionality constant (k_2). In this case k_1 is actually a reflection of the Michaelis kinetic parameters V_{max} and Km ($k_1 = V_{max}/Km$). The proportionality constant, k_2, is specific for each xenobiotic chemical, and its value is assumed to be the same for all microbial communities, regardless of their size, activity, or source (site-independent). This suggests that the biochemical kinetic constants for degrading any one xenobiotic chemical are essentially "universal."

The Athens model, however, suffers from several practical constraints and inadequately tested assumptions. Most obvious in this respect is the term in the expression which requires information on the actively degrading biomass. Several biomass measurements have been used in attempts to test this model. Measurements of CFU, AODC counts, LPS measurement, ATP, etc. have found limited utility with relatively few substrate classes in only a few habitats. To be sure, there is general disagreement on the "best" biomass measurement currently in use.

In addition, the derivation of the model assumes that substrate concentration is much less than K_m. Since many laboratory experiments use starting substrate concentrations in the range of 20-200 µg/l, it is assumed that K_m must therefore be relatively high. To ensure that degradation rates are measured in the first-order region, substrate concentrations should be at least one-tenth the K_m value. Practically, this means K_m values should be in the low µg/l range. Thus, it is quite possible that a biodegradation rate determination might be unknowingly performed in the zero-order region rather than the first-order region. Before substrate concentrations can be assumed to be well below K_m, an indication of the magnitude in K_m for different xenobiotic chemicals must be obtained. The implicit assumption that K_m values are constant relative to other parameters such as V_{max} also needs to be tested.

ACTIVITY MEASUREMENTS

It is apparent in the development and use of biodegradation kinetic models that (a) the activity of a microbial community (which includes different bacterial populations), (b) the relative concentrations of the active bacteria, and (c) the environmental factors controlling their activity need to be assessed using a minimum number of parameter estimates. The more encompassing and integrative the parameter estimates, the less are required. We believe that EPA should support research to develop activity measurements that are consistent with known biological theory, easily performed by a variety of laboratories, and applicable to a wide variety of xenobiotic chemicals and ecosystems. The initial use of total viable cell numbers in a microbial community was, in essence, an attempt to provide an "activity" measurement. It was assumed that all bacteria, or some constant proportion thereof, were active in degrading a chemical and that they were all equally active. Evidence presented at this workshop and from other sources indicates that this "activity" measurement is apparently applicable to a limited number of chemicals and ecosystems. This is due in large part to problems in the original assumptions. Similarly, measurements of the numbers of specific chemical degraders within a population are limited because of methodological problems and the realization that the mere presence of a degrader does not necessarily mean it is active. Thus, new approaches for relating the activity of a microbial community to its degradative potential are required.

Two promising approaches are the measurement of the maximum velocity (V_{max}) of degradation and the first-order decay rates (k_1).

In the saturating or zero-order region of substrate decay, the biodegradation rate (V_{max}) is limited by the active catalyst concentration and not by the substrate concentration. Therefore, the rate observed at saturating substrate levels is a direct reflection of the actively degrading biomass. This is exactly the quantity required in the Athens model (that is $k_2 = k_1/V_{max}$), but it uses a rate of biodegradation instead of cell number. At first glance, it would appear that the units for this proportionality constant are unusual (i.e., liters.µmoles^{-1} or reciprocal of K_m units). However, when this kinetic constant is properly employed, -dS/dt will have recognizable units (i.e., µmoles.l^{-1}.h^{-1}).

The first-order region of substrate decay is also a reflection of activity degrading biomass when the substrate is present at concentrations well below the K_m of the reaction. In this region, a constant fraction of substrate undergoes conversion to product at any given moment, and the absolute velocity decreases as the substrate is consumed. Thus, the rate for a given reaction is substrate dependent.

It is important to note that first-order kinetics will often be displayed if some other factor mitigates the consumption of a substrate by the requisite microorganisms. For instance, this behavior will be observed if biodegradation is limited by diffusion of the molecule to the site of microbial activity. Such phase transfer resistance is common for the consumption of gaseous substrates or possible for chemicals that tend to partition strongly to solids.

Both approaches (V_{max}, k_1) have minimal interpretation difficulties, and each is a direct measure of activity. Secondly, they are not limited to a particular type of activity (e.g., hydrolysis, hydroxylation, deamination, etc.), and they are not restricted to a given chemical class or series of structurally related compounds. Both approaches are also independent of the nature of the catalytic unit. It matters little if the biotransformation is actually catalyzed by an extracellular enzyme, a bacterium, a fungus, or an anaerobic consortium of organisms. The approach can be used in a variety of environments, including single phase systems (i.e., water alone) or multiphase systems (i.e., sediment/water slurries). In addition, the activity measurement will already reflect an integration of the environmental factors influencing biodegradation. In general, V_{max} determinations do not require the analytical sophistication associated with the measurement of low substrate concentrations; as a corollary then, the requirements for radiolabeled material may largely be circumvented. Biodegradation rates obtained at saturating concentrations of substrate (V_{max}) permit subsequent predictions of rates at any concentration (assuming the Athens model is essentially correct). Biodegradation rates obtained in the first-order decay region are only predictive at lower substrate concentrations. Finally, the suggested activity measurements can be made by all laboratories using relatively simple incubation systems.

METHODOLOGY

Ideally we would like to see research designed to give all the salient kinetic parameters (K_m, V_{max}, k_1, and k_2) in a single experiment or set of experiments. It is desirable to get the maximum amount of pertinent information from a limited amount of experimentation, particularly when the inoculum tends to be difficult to obtain. Based on the information presented at this workshop, it would appear that there are two promising methods to accomplish this goal: (a) progress curve experiments and (b) experiments generating typical substrate concentration-velocity data pairs. Both approaches have advantages and disadvantages, and neither is applicable to all chemicals in all environments. The techniques should be chosen with special regard to practical constraints such as the ease of obtaining the measurements and of gaining the maximum amount of information with a minimum of inoculum, time, effort, and cost.

1. Progress curves. Progress curves are experiments designed to follow substrate depletion or product formation through the zero-, mixed-, and first-order regions. By analyzing these experiments using either nonlinear

parameter estimation techniques (as recommended by the statisticians) or linearizing transformations of the data, the various Michaelis constants V_{max} and K_m can be obtained, and thus k_1 and k_2 can also be calculated. *The real advantage to this technique over others is that the kinetic parameter estimates can be derived from a single experiment*. Progress curve experiments also lend themselves to manipulating environmental factors (i.e., partitioning, temperature, etc.) which will help describe quantitatively the influence of these factors on biodegradation processes.

A limitation of this technique may be the difficulty of obtaining saturation concentrations for some substrates. However, this remains to be seen. If the degradation of xenobiotic compounds is enzymatically catalyzed, saturation kinetics should be observed.

Obviously, we realize that progress curve experiments are not designed as simulations of complex environments. Rather, they are tools to help us understand the intricacies of nature with an eye toward predicting biodegradation kinetics.

Unlike other types of experiments designed to estimate biodegradation rate parameters, the assumptions in this experimental approach are straightforward. Simply stated, decreases in reaction velocity with time are due only to decreasing catalyst saturation, and the biodegradative process is unlinked to significant changes in catalyst concentration over the course of the experiment.

The latter assumption presumes that the particular biodegradation process is unrelated to significant microbial growth. Of course, this assumption will not always be the case, and microorganisms will proliferate as a result of the metabolism of a xenobiotic carbon and energy source. However, we feel that the recommended approach and ensuing rate estimations are basically conservative. There will be a certain amount of error associated with any rate estimation, and from an environmental policy viewpoint, we feel that it is better to err on the conservative or low side rather than to overestimate the natural biodegradation rate. If microbial growth does occur as a result of xenobiotic compound utilization, this ultimate expression of metabolism will be reflected in an adaptation phenomenon (see below), and rate estimations can be periodically updated.

2. *Substrate concentration-velocity experiments.* The same kinetic parameters can also be obtained by performing typical substrate concentration-velocity experiments and analyzing the results by nonlinear regression or linearizing techniques. These experiments involve determining the initial rate of substrate disappearance at several different substrate concentrations. Either labeled or unlabeled substrates can be used, depending on analytical sensitivity required. Typically, such experiments assume that the reaction proceeds to a negligible extent during the course of the assay (i.e., less than 5% substrate depletion). Under some and perhaps most experimental conditions, it may not be possible to restrict the reaction to this extent. At best, these experiments usually employ 5 or 6 data points. This limitation is not inherent in progress curve analysis, since the Michaelis-Menten expression is a differential velocity equation that is valid over the entire concentration range and as many data points as desired can be incorporated into the analysis.

Several important factors may limit the widespread applicability of the substrate concentration-velocity method. First, radiolabeled substrates are often required to detect very low concentrations or small changes in substrate concentrations against a large background. Considering the myriad of chemicals and their potential availability and cost, it is unlikely that techniques relying on radiolabeled materials will gain widespread acceptance.

Secondly, parameter estimations from substrate concentration-velocity data pairs often rely on linearized plotting techniques. Good examples include heterotrophic activity experiments, which usually use plots analogous to the double reciprocal plot of Lineweaver-Burk. However, to obtain accurate parameter estimates using this approach, some a priori knowledge is required, since the chosen substrate concentration range is critical and must be in the neighborhood of K_m. If the chosen substrate range is too high (relative to K_m), the observed velocity is insensitive to changes in substrate. Thus V_{max} can be accurately estimated, but K_m will be inordinately low. If the chosen range is much lower than K_m, the curve will intercept the graph axis too closely to the origin to allow accurate K_m or V_{max} estimates (K_m and V_{max} will be very large). The substrate increments chosen can also critically influence parameter estimation. Other techniques of plotting substrate-velocity data have similar ideal concentration ranges. As a corollary to the above cautions, saturation is almost never checked in these experiments to verify that one is truly in the appropriate concentration range. Nevertheless, if appropriate concentration ranges are chosen, accurate parameter estimation can be made using this technique.

Lastly, from a practical standpoint, many substrate concentration-velocity experiments rely on CO_2 formation and detection. The assumption is that primary degradation is the rate limiting process and that subsequent conversion to CO_2 is relatively rapid. However, even a cursory review of the literature reveals that CO_2 may not represent the ultimate fate of the test substrate. Primary degradation reactions may yield intermediates that can bind, sorb, or are generally more persistent than, starting chemicals. Thus, before experiments are designed, CO_2 formation must first be correlated with substrate disappearance.

RESEARCH PLAN

MODEL VALIDATION

Research effort should continue to test further the usefulness of the Athens model. We need to determine if it is desirable and feasible to obtain a k_2 proportionality constant for every xenobiotic chemical (or class of chemical if the benchmark approach is utilized). Research to date has shown that the use of total numbers of bacteria as the normalizer for different degradative activities among microbial populations has been partially successful. Some compounds that are apparently degraded by hydrolytic mechanisms give consistent k_2 values when the first-order rate is divided by total cell numbers. However, further work along these lines is not recommended for two reasons. First, there does not seem to be a consistency trend indicating which chemicals are amendable to the total cell number approach. For example, some chemicals that are hydrolytically attacked do not give consistent k_2 values while others do. Secondly, since the activity of microbial communities is unpredictably related to cell numbers, the conceptual basis behind using total cell numbers is obscure.

We believe, as we have indicated above, that a more direct measure of microbial activity is needed to obtain and compare k_2 values. To determine which activity measure to use, we recommended that a decision be made relative to the magnitude and variability in values of the Michaelis half saturation constant, K_m , for a variety of xenobiotic chemicals. In other words, assumptions about K_m used in the formulation of the Athens model must be carefully and thoroughly tested. There is currently a paucity of kinetic information upon which to make such decisions. We feel strongly that this is where the research effort in EPA should be focused.

The conceptual framework for a research effort involving the Michaelis half-saturation constant is shown in Figure 1. The K_m values for xenobiotic chemicals can be relatively high or low. Enzymes capable of metabolizing pollutant chemicals that closely resemble native substrate(s) would be expected to exhibit high affinities for the xenobiotic compound and thus low K_m's. Chemicals totally foreign to the environment would probably have enzyme systems with relatively high K_m values. If K_m is consistently high for most xenobiotic chemicals, then an adequate measure of microbial degradative activity for any environmental sample would be the first-order decay constant (k_1). This would be an integrative measure representing the inherent rate at which the catalytic unit(s) would attack the chemical, the number of catalytic units involved, and the factors that control catalysis. Knowledge that K_m was always high (i.e., mg/l range) would mean that laboratory experiments typically using µg/l quantities of the substrate would rarely produce erroneous k_1 estimates.

If K_m values are consistently low (µg/l range) or widely variable, it could not be assumed that a measured degradation rate will necessarily reflect first-order decay. The mere observation of apparent linearity in a log-linear plot of substrate concentration versus time does not ensure first-order decay. In fact, the substrate concentration can decrease, as observed on a semi-log plot, by over two orders of magnitude and still be in the zero-order region of the decay curve (see illustrations presented in papers by Suflita and Lewis and Hodsen). A zero-order rate, or maximum velocity (V_{max}), if misconstrued to be a first-order rate, can lead to erroneous environmental predictions. The kinetic values V_{max}, K_m, k_1, and k_2 should therefore be determined in experiments using substrate concentration-velocity data pairs or progress curve techniques.

The constancy of the K_m values relative to V_{max}, whether they are high or low, must also be determined. One can reasonably anticipate that V_{max} values, as reflections of the active catalyst concentration, will vary depending on the habitat examined. For instance, one might expect a small catalyst concentration (V_{max}) in an oligotrophic stream relative to one that received an input of the xenobiotic chemical in question. However, the mechanisms evolved for the metabolism of such compounds may be limited. This would then be reflected in the affinity of the enzyme for the substrate in question. In other words, if the K_m varies randomly by orders of magnitude from one microbial community to another, then the development of a generalizing site-independent kinetic model is very unlikely. A kinetic description for biodegradation would then require much more detail about the site-specific factors that control the rate.

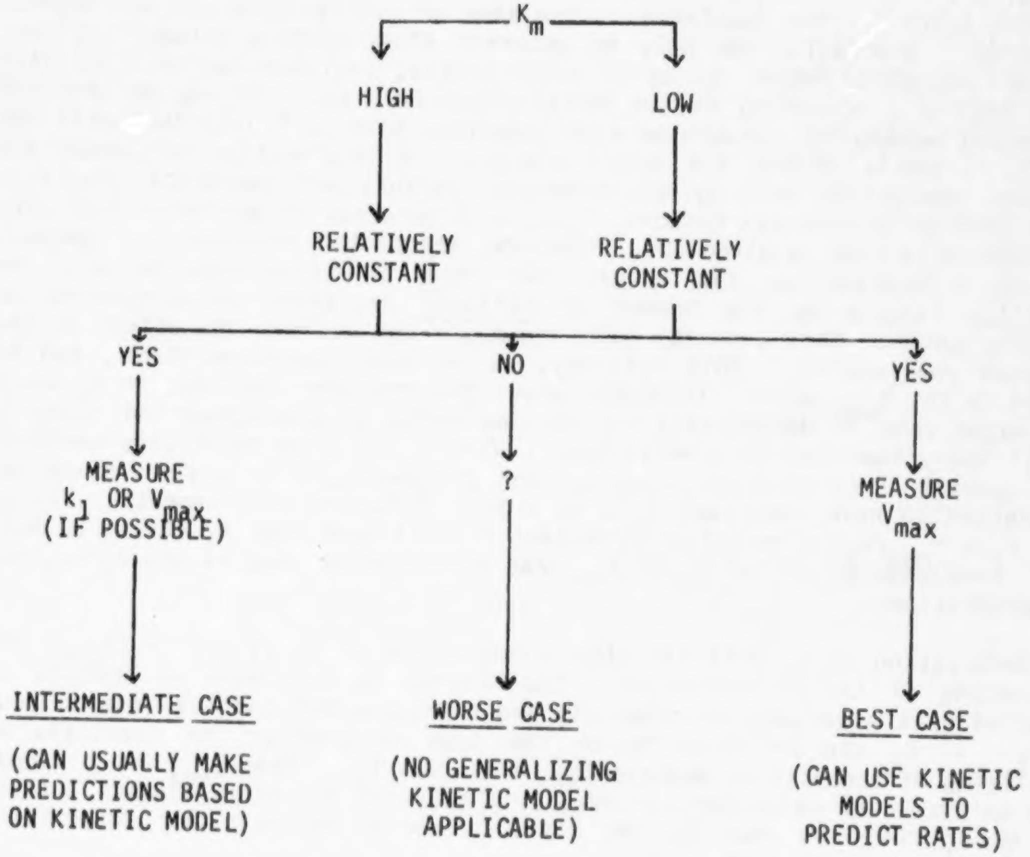
K_m
HIGH
LOW
RELATIVELY CONSTANT
RELATIVELY CONSTANT
YES
NO
YES
MEASURE k_1 OR V_{max} (IF POSSIBLE)
?
MEASURE V_{max}
INTERMEDIATE CASE
(CAN USUALLY MAKE PREDICTIONS BASED ON KINETIC MODEL)
WORSE CASE
(NO GENERALIZING KINETIC MODEL APPLICABLE)
BEST CASE
(CAN USE KINETIC MODELS TO PREDICT RATES)

Figure 1. Flow diagragm for research strategy in testing kinetic models of biodegradation.

If K_m values for xenobiotic chemicals are reasonably constant relative to V_{max} values, then three hypotheses follow. First, the average affinity of the catalytic units for the substrate is the same or similar from one environment to another. Similarity may only be apparent after certain categorizations, i.e., oligotrophic versus eutrophic water bodies, polluted versus unpolluted areas, naturally occurring versus exotic chemicals, etc. It may be that the biochemical mechanisms associated with substrate transport into the cells and initial catabolic attack are quite similar. Biodegradation processes are therefore controlled more by environmental factors and substrate chemistry rather than by biochemical factors, i.e., a large array of metabolic machinery has apparently not evolved to transform xenobiotic chemicals. Second, assuming biodegradation can occur, the rate of transformation will be controlled largely by the number of actively degrading microorganisms or catalytic units. This activity will encompass and reflect the effect of the immediate environment. This activity, as we have suggested above, can be equated to the V_{max} value. Knowledge about the relative constancy in K_m would be a major step in determining if the complexity of biodegradation rates in natural ecosystems can be generalized. If K_m's are also relatively constant for a general chemical class, then perhaps all chemicals in a given class can be expected to have invariant K_m's in widely different environments. Third, since $k_1 = V_{max}/K_m$, a relatively constant K_m will mean that k_1 varies as does V_{max}. Knowledge of either k_1 or V_{max} can therefore be used in biodegradation rate predictions.

Information on K_m will facilitate derivation of k_2 values (k_1/V_{max}) and the testing of the Athens model. Experiments to determine k_2 need to be conducted with a variety of chemicals and environmental samples from diverse areas. If k_2 can be shown to be the same or similar for chemicals by normalizing differences in degradation rates with V_{max}, then V_{max} would appear to be an excellent parameter for assessing actively degrading biomass. Thus, V_{max} or k_1 could be used as the basic parameter estimate for determining criteria for extrapolation.

ESTABLISHING KINETIC PARAMETERS

To be sure, there is a limited amount of information on the basic kinetic parameters of K_m, V_{max}, k_1, and k_2 governing pollutant biodegradation. Our current thoughts on biodegradation kinetics are an integration and a direct outgrowth of the information presented at the workshop. We feel that in order to test models for biodegradation kinetics, efforts using judiciously selected model substrates in a number of habitats should be expanded toward establishing the parameters mentioned above.

The criteria for selection of the model substrates include: (a) their potential for analysis, (b) their high water solubility, (c) their lack of significant sorption, (d) the availability of radiolabeled compounds, (e) the amount of background information on their biodegradation, (f) their ability to be metabolized aerobically and anaerobically, (g) representativeness of different chemical classes, and (h) their potential for exhibiting a relatively wide range of biodegradation rates. A potential list of chemical types that could be tested is presented in the workshop report. In addition, we suggest that the initial efforts start with three substrates: 4-nitrobenzoate, 4-cresol, and 2,4-dichlorophenoxyacetic acid. These compounds should be tested by establishing their kinetic parameters (K_m, V_{max},

k_1, and k_2) in habitats ranging from oligotrophic environments to eutrophic ones. For example, biodegradation in a polluted portion of an estuary may be compared with a pristine area. Another example would include freshwater lakes of differing (but well defined) trophic status. These habitats as well as others (sewage, sludge, streams, rivers, open ocean, groundwater, etc.) should be used to help determine if predictive kinetic models of biodegradation are truly feasible.

The techniques used in this effort should include the shake flask, since it is this method which is the most likely to be used on a widespread basis. The design and analysis of these experiments will be those described earlier in this document. Degradation studies with suspended sediments should also be included.

ENVIRONMENTAL FACTORS AFFECTING BIODEGRADATION

As pointed out by the workshop panel report, there are many factors that can serve to accelerate or retard the biodegradation of xenobiotic susbstances. Considering the multiplicity of these factors, one questions whether or not it is expeditious for EPA to support research to quantitate the influence of these factors.

Our approach to assessing metabolic activity by measuring laboratory biodegradation rates seems to be a way of accounting for these environmental factors without actually knowing what they are. For instance, such results will tell us if a biodegradation rate is relatively slow or fast. Even though such experiments will not allow us to conclude why this is so. We feel that by measuring rates we can successfully integrate the many environmental factors influencing biodegradation and can limit the number of false positive indications of biodegradation.

It is fully recognized that it is desirable to learn as much as possible about the factors controlling biodegradation rates in the environment. However, we believe that pragmatically a detailed knowledge of these factors is not necessary for rate predictions. As more and more factors become elucidated, quantitative relationships describing their influence on biodegradation will be established. The basic biodegradation kinetic model will thus grow in degree of sophistication to accommodate these relationships. However, for each new factor described, field information on when and where that factor will have its effect must be obtained to make more sophisticated predictions. The subsequent monitoring effort will increase exponentially with each new factor or concern. Our philosophy suggests that research efforts must initially be oriented in the direction of finding parameter estimates that integrate the effect of these environmental factors into a small number of measurements. We feel that either V_{max} or k_1 measurements lend themselves to this goal and therefore should be strongly emphasized. If this concept does not eventually prove useful, we will perhaps have to investigate more intensively the factors that control biodegradation rates in order to find a better quantitative basis for environmental predictions of biodegradation rates.

Work should also continue on the influence of environmental factors, because the resulting information will eventually help extrapolation research (see next section). It may also provide the basis for experiments that attempt to manipulate the environment to improve biodegradation capacity or rates for the purposes of eliminating certain in-place environmental pollutants. Dr. Raymond's paper describing the use of nutrient additions to accelerate hydrocarbon degradation in ground water is an excellent example.

ADAPTATION

Numerous published investigations and extensive discussions at this workshop have indicated that many xenobiotic chemicals do not degrade immediately when added to an environmental sample. A lag or adaptation period of varying lengths is observed before a chemical is rapidly degraded. The extent of the adaptation period directly affects the exposure of the biota to the pollutant. The incidence of adaptation and the mechanism(s) controlling adaptation should therefore be as extensively studied as biodegradation rates. Therefore, lag phases must be considered in possible regulatory decisions.

At this point, the length of time before the onset of rapid degradation is impossible to predict since the phenomena is poorly understood. Therefore, it is difficult to model this portion of a decay curve. Probable reasons for adaptation include the requirement for bacterial growth before significant parent chemical is transformed, specific substrate concentrations before the requisite enzymes are inducted, the need to deplete competing substrates before the compound of interest degrades, or the need to exchange the essential genetic material. Other mechanisms of adaptation can certainly be postulated.

At present, a generalizing descriptive model for adaptation has not been proposed. This is largely because of an absence of information on the mechanisms controlling adaptation. However, the adaptation event itself can be readily observed experimentally. Adaptation, or the length of time before accelerated biodegradation occurs, is an integrating parameter which, like the V_{max} value, does not require a knowledge of the exact adaptive mechanisms or the factors controlling it. We therefore believe that research efforts should investigate the length of the lag or adaptation period. Three major approaches should be considered. First, the effect of substrate concentrations on the length of the lag period should be cleary defined for a variety of chemical types and environmental samples. Arguments that adaptation will not occur at environmentally realistic concentrations (μg/l) must be experimentally substantiated. Second, the relationship of adaptation to the growth of organisms capable of degrading the substrate must be investigated. This effort is needed to establish whether lag periods are simply a function of the initial number of degraders in the environmental sample and their eventual growth rate in that sample. This assumes that if the degraders are present they will commence growth immediately following exposure to the substrate. Examples where degraders are present but never grow are assumed for now to be infrequent and may consequently form the basis for research in the future. If growth can be quantitatively linked to the lag period prior to adaptation, the development of descriptive models becomes feasible.

Third, the natural variability in adaptation time should be examined to determine if regulatory decisions can possibly be based on certain consistencies in the adaptation response. For example, will the adaptation time for a specific xenobiotic chemical always be the same if environmental samples are always taken from the same general area or does the adaptation time always vary widely in time and space? If consistencies can be observed, it will be important subsequently to relate them to adaptation mechanisms, chemical structure trophic conditions, topographic and hydrogeologic features, and previous exposure to pollutants.

EXTRAPOLATION

Extrapolation of laboratory data to the field is a simple concept that is poorly defined. Extrapolation means different things to different investigators, and a clear plan that outlines the research questions and suggests practical, experimental approaches does not exist. In an effort to correct this situation, we propose a research strategy that would involve: (a) establishing the "environmental relevance" of laboratory-derived data, and (b) categorizing the variation in biodegradation rates in natural environments in such a manner to permit predictions of exposure concentrations.

ENVIRONMENTAL RELEVANCE OF LABORATORY DATA

It is frequently argued that xenobiotic chemical biodegradation rates obtained from simple laboratory tests (e.g., river die-away, shake flasks, etc.) are environmentally insignificant since, by design, these tests replace the complexity associated with natural environments with expediency, standardization, and reproducibility. Laboratory tests are important for regulation and chemical registration, but it is not clear how accurately this information reflects what actually happens in the field. To be sure, industry cannot examine every chemical in a laboratory system that simulates real world conditions, but information can be supplied that will delineate the qualitative and quantitative degree to which a simple laboratory method represents field events. Simple awareness of the deficiencies of a laboratory test may be very useful from a regulatory or extrapolation viewpoint. Modifications of existing tests or the addition of supplemental tests will likely be the most productive outcome of this type of relevancy study. Such modifications will greatly improve our confidence in making fate predictions from laboratory information.

We propose that the environmental relevance of laboratory test data can be most efficiently determined using microcosm studies and field studies. Based on recent information, microcosms appear to provide good simulations of the field. They can be dosed indiscriminately, and they are isolated from uncontrollable weather conditions. They certainly provide a means of studying biodegradation processes under complex conditions. Microcosm studies therefore, in our opinion, are the methods of choice for such relevancy studies. Whenever possible, the fate of a xenobiotic chemical should also be examined under field conditions. Only experience in both types of studies will determine our confidence in and limits to the the use of microcosms.

A research plan involving microcosms and field studies would consist of using biodegradation information from simple laboratory tests to see if it can adequately explain the biotic fate of a chemical in more complex ecosystems such as microcosms or field studies. If this hypothesis proves correct, then we believe that the environmental relevance of the laboratory test data will have been established.

If it cannot, then manipulation of the complex system or the isolation of component parts thereof may indicate how to modify appropriately the simple laboratory test so that it will accurately describe natural events. In some cases, complexity may control biodegradation rates to such an extent as to prohibit reduction to simpler terms. If this is the case, a routine and standardizable complex laboratory test system must eventually be developed and tested.

The use of laboratory data to explain events in complex systems is complicated by the fact that biodegradation will not be the only fate process affecting a chemical. Dilution, sorption to sediments, diffusion, bioturbation, bioaccumulation, photolysis, hydrolysis, volatility, etc., may also influence the fate of the chemical. Recent investigations indicate that the use of appropriate controls, physical and chemical tracers, unique analytical techniques, and simple mathematical models will permit a determination of the biotic fate of a chemical in a complex system and will allow a comparison with information generated in simple laboratory tests. We recommend that these research efforts be continued and expanded. Test chemicals have to be selected carefully, and ancillary experiments will have to be carried out for additional information. Microcosms that simulate specific portions of ecosystems need to be developed and tested. Appropriate field sites will have to be located. Dosing, analytical, and data analysis procedures need to be developed.

We feel strongly that microcosms and field studies have relatively little predictive value. They are generally too small to be truly representative of any large ecosystems. They are instead excellent validation tools. Kinetic models for biodegradation, sites of active biodegradation, and factors that control biodegradation rates can be tested in these systems and their environmental significance determined.

VARIATIONS IN BIODEGRADATION RATES

Estimating biodegradation rates using laboratory studies requires, as we have proposed above, the determination of V_{max} value (assuming a need for a site-independent kinetic constant k_2) or the first-order rate constant. Environmental samples are usually collected from a field site for this purpose and then used in a laboratory biodegradation test system. The resulting estimation of biodegradation, k_1, or the activity of the microbial community (V_{max}) is representative of that site only. Its representativeness of the rest of the ecosystem is unknown. The values are likely to vary widely, depending on when and where the sample is taken. Little appreciation for this natural variation currently exists. Is it so extensive as to force us to analyze biodegradation rates over very small spans of space and time? Perhaps biodegradation rates or the activities of microbial communities responsible for the biodegradation are not as widely varying as we suspect. Or perhaps variability can be categorized relative to certain general charactristics of the ecosystem. Will eutrophication tend to decrease or increase variability? Will different degrees of pollution or prior exposure to xenobiotic chemicals affect variability, or will the absolute rates just increase or decrease? Thus, we believe that an extensive research effort should be centered on measuring the variability in biodegradation rates. This appears to be the only way that we will be able to truly extrapolate laboratory data to the field, to extrapolate biodegradation information from one environmental site to another, and to extrapolate biodegradation information about one chemical to another in the same chemical class.

We suggest that the temporal and spatial variability of k_1 or V_{max} in one typical ecosystem (estuary, river, lake, etc.) be established. Statistical designs, which are readily available and adequately tested, will have to be employed for the most efficient estimation of this variability. In that ecosystem, the largest area that will consistently give biodegradation rates within a pre-specified range must eventually be determined. In an estuary, for example, the area considered might include the water (at an average depth) and sediment for the main embayment. Determination of the range will be based on the actual observed variability, on the toxicological properties of the chemical, and on certain ecosystem characteristics. The objective will be to determine the frequency and size of "hot spots" or "low spots," if any. The range in biodegradation rates tolerated will essentially control the significance of hot spots. For example, one portion of the main embayment in an estuary may consistently give degradation rates of V_{max} values that fall outside the observed variability in the rest of the embayment. This hot spot may not, however, be significant if one is willing to accept the additional variability. Acceptance or nonacceptance will again depend on the observed data, the chemical being tested, and certain regulatory considerations. Efforts must then continue to identify hot spots in other corners of the ecosystems and during other seasons of the year. The maximal use of statistical design will be crucial for optimized determination of variability.

With an estimate of the variability for the biodegradation of one chemical in one ecosystem at hand, cluster analysis of the data should be performed to determine the minimum number of measurements necessary to detect similar variability in any new ecosystem. If the number is too large then the variability in degradation rates in aquatic environments is too extreme for us to make generalizations over large areas within a ecosystem. Degradation rates become very site specific, and extrapolation of laboratory data to the field is unfeasible. However, if the number of required measurements is reasonable, then examination of the temporal and spatial variability in other ecosystems should be carried out.

We propose that two or three geographically different field sites be chosen for each major ecosystem type (e.g., rivers, estuaries, streams, lakes, etc.) to measure the variability in k_1, V_{max}, or K_m. For example, two or three different lakes could be selected in different geographic areas to represent this ecosystem type. The selection would be based, in part, on accessibility for experimental purposes and, in part, on environmental characteristics of the field site. We believe that initially field sites representing extremes (i.e., polluted versus pristine, eutrophic versus oligotrophic) should be examined for their variability. If variability is "similar" for each of these extremes, then it can be argued that site-to-site extrapolation is feasible within certain limits of error. A chemical, for example, may show an average half-life of X days plus or minus some error term for one particular site of bounded environment. If variability can be assumed to be the same from one site to another, the average half life for a chemical determined at a new site can be assigned the same variability as the original site.

The large number of chemicals examined by OTS precludes an assessment of variability in biodegradation rates for each new chemical. Some form of structure activity relationship is obviously necessary. We propose that the biodegradation rates of structural analogues within a given chemical class can be ranked from the fastest to the slowest. The consistency of the ranking(s) should then be examined using samples taken from several field sites at different times. If one were to examine the chemical class of chlorinated biphenyls, for example, would monochlorinated biphenyls always degrade faster than dichlorinated biphenyls, regardless of the source of the inoculum used in the biodegradation experiments? Would 4,4-dichlorobiphenyl always degrade faster than 3,4-dichlorbiphenyl, etc.? A consistent ranking would prove invaluable. It would mean that the variability in biodegradation rates in time and space for one representative of a chemical class (i.e., a "benchmark" chemical) could be linked to other chemicals of that class through the ranking analysis. Consistent rankings may only be possible if different subsets are established. Ranking using samples from polluted areas may be different from those using samples from nonpolluted areas. Extensive eutrophication may also result in different rankings. Likewise, rankings using samples from river water should not necessarily be expected to agreee with those using estuarine samples.

We suggest that the major chemical classes of concern to OTS and EPA be identified and a representative member of each class be selected. The partial list of chemicals discussed at this workshop is an excellent start in this direction. Work can then begin to establish consistencies in the rankings of their biodegradation rates with other chemicals within those classes.

Appendix: Proposed Benchmark Chemicals for Biodegradation Research

The following chemicals were recommended by an ad hoc committee who met briefly on the last day of the workshop:

1. pentachlorphenol
2. p-nitrobenzoate
3. 2-mercaptobenzothizole (MBT)
4. pthalate esters
5. phenylcarbamate or thiocarbonate
6. decalin or phenanthrene
7. phenoxybenzoate
8. ABS
9. carboxyazobenzene (or similar)
10. polyethylene glycol
11. Dalapon
12. tertiary or quadamine (EDTA or NT)
13. cyclohexane carboxylic ac.
14. substituted pyridine
15. parachlorobiphenyl

The criteria for choosing a benchmark chemical were:

1. Range of physical properties (e.g., anionic, cationic, hydphobicity)
2. Range of chemical structure (e.g., heterocyclic - aromatic and aliphatic branched, polymeric, straight chain)
3. First-order fate process -- biodegradation
4. Variety of initial reaction mechanisms
5. Safety in handling
6. Availability and cost
7. Environmental relevance

The purpose of using benchmark chemicals was to compare laboratory test systems and to test the integrity of those systems.